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Double-Stranded Ribonucleic Acid Killer Systems in Yeasts

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INTRODUCTION

Killer yeasts secrete polypeptide toxins which kill sensitive cells of their own species and frequently those of other species and genera of yeast (Fig. 1). This phenomenon has been observed in many of the yeast genera investigated, but its prevalence varies widely among natural isolates (111, 116, 130, 170). Although a majority of natural isolates apparently lack both toxin production and the associated specific toxin immunity, this must be interpreted cautiously, since assays for both toxin production and immunity require choice of an appropriate sensitive strain and determination of optimal conditions for toxin activity. Nevertheless, it seems that, whereas toxin production is probably important in yeast ecology, the ecological advantage conferred by toxin production is seldom paramount, or their determinants are unstable, or such production is associated with deleterious effects. Although this uncertainty reflects a lack of data on the persistence of killers in the wild, no deleterious effects are known, and their genetic determinants are stable in the laboratory. Low prevalence may indicate that yeast killers are inefficient assassins. Their determinants might often be maintained as parasitic passengers rather than as selected, offensive machinery essential to survival in internecine warfare.

In the few instances where killer determinants have been identified, they are cytoplasmically inherited (7, 46, 127), double-stranded (ds) RNA (4, 6, 95, 142) or DNA (53) plasmids which encode toxin precursors (11, 51) and which are dependent on a similar plasmid for maintenance. The basis of this dependency is known only for the *Saccharomyces cerevisiae* killer plasmid M-dsRNAs. These dsRNAs are found only in cells also containing an additional dsRNA species called L. Both types of dsRNA exist in cytoplasmic virus-like particles (VLPs) (22, 58), and both dsRNAs require a protein encoded by the L-dsRNA (62) for encapsidation (13, 55). Killers are a minority among natural *S. cerevisiae* isolates, although most strains contain L-dsRNAs. Such strains are sensitive nonkillers. The helper L-dsRNA plasmids, unlike the killer determinants, have no known dependence on other plasmids, confer no known phenotype on the host cell apart from their effects on M-dsRNA maintenance, and, when alone, appear to exist as truly parasitic entities.

Clear parallels exist between the secretion of yeast killer toxins and the production of bacteriocins. Production of both is associated with specific immunity, and toxicity is restricted to similar cell types. Killer yeast strains might, therefore, more properly be called mycocinogenic strains and their toxins, mycocins. However, the original nomenclature is now well established and, as pointed out by Bussey (23), has helped to bring an esoteric aspect of yeast molecular biology to the attention of a broad range of scientists.

In this review we will focus on the molecular biology of the most intensively investigated killer system, the M_1-dsRNA-determined K1 killer system of *S. cerevisiae*. We hope to demonstrate the relevance of this system to many of the vital issues in yeast and general eucaryotic molecular biology.

It should be emphasized that cytoplasmic dsRNA species have been shown to determine the killer phenotype only in *Saccharomyces* sp. and *Ustilago* (73) sp. strains. Only in one other species, *Kluyveromyces lactis* (51), has cytoplasmic inheritance of the phenotype and the nature of its determinant (one of two linear dsDNA species [53]) been clearly demonstrated. Brief summaries of recent information on the *K. lactis* and *Ustilago maydis* systems are included, because of their comparative value and intrinsic interest.

KILLER dsRNAs, MYCOVIRUSES OR ENCAPSIDATED PLASMIDS?

The existence of cytoplasmic VLPs containing dsRNAs was well established in the mycoviruses of filamentous fungi (85, 86) well before the discovery in 1963 (M. Makower and E. A. Bevan, Proc. Int. Congr. Genet. XI 1:202, 1963) of the killer phenotype in laboratory strains of *S. cerevisiae*. These mycovirus VLPs were found to contain a limited number of capsid proteins and an associated dsRNA transcriptase activity (21) and to consist of a population of individually encapsidated, apparently interdependent dsRNA species. These properties led to their designation as fungal "viruses."

In considering interactions between the multiple dsRNA components of mycoviruses, it should be remembered that, whereas the Reoviridae and other dsRNA viruses of higher eucaryotic cell types (reviewed in reference 36) contain a single copy of each of their component dsRNA segments in each of their virus particles, ensuring equimolar representation, mycovirus dsRNAs are under no such numerical restraint, so that differential demand for gene products can be reflected in the relative copy numbers of the relevant dsRNAs.

When the yeast killer phenotype was shown to be associated with particular dsRNA species (M_1, etc.) (6, 95, 131, 142), which were later shown to be present in cytoplasmic VLPs (22, 58), it became clear that the killer phenotype was an attribute of a yeast mycovirus which, following the established nomenclature, was called the *S. cerevisiae* "Virus," ScV (62). The VLPs containing various M- and L-dsRNA species can, therefore, be designated ScV-M_1, ScV-L_{1A}, etc., since reciprocal interdependence between these species apparently does not exist (see below, "Role of L-dsRNAs in VLP Capsid Production and M_1-dsRNA Maintenance").

No mycovirus, including the yeast killer system, has been shown to be capable of extracellular transmission. One recent report of transmission of the dsRNAs of *Helminthosporium victoria* would be an exception, if confirmed (see below, "Effects of dsRNA Mycoviruses in Pathogenic Fungi") (48). On the contrary, mycoviruses and the killer dsRNAs are stably maintained, at relatively constant copy number, by vertical transmission. Transmission occurs only by cytoplasmic mixing during budding, mating, or other natural or artificial forms of cell fusion. The *S. cerevisiae* dsRNA killer determinants may thus be regarded as virus-like plasmids. They clearly possess properties relevant to both the truly infectious dsRNA viruses (36) and to plasmids of eucaryotic cells. Their genetic analysis resembles that of multicopy cytoplasmic plasmids, and for the best-studied species (M_1-, M_2-, and L_A-dsRNAs), their dependence on multiple nuclear genetic loci clearly demonstrates their lack

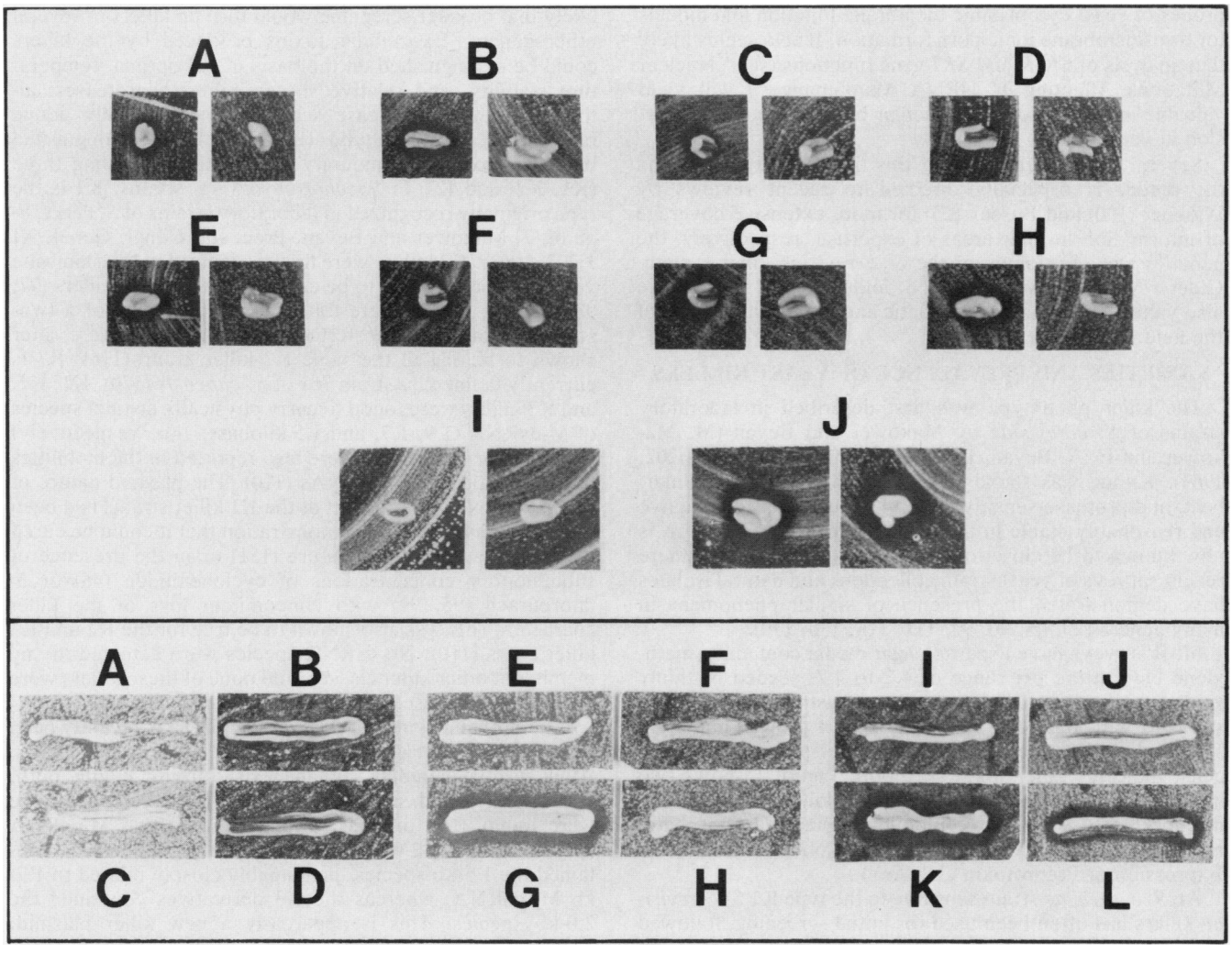

FIG. 1. Expression of killer and immune phenotypes, in standard plate assays, of a standard killer containing M_1-dsRNA and of transformants containing M_1-cDNA. (Upper) To test killer activity, strains were patched onto a lawn of *S. cerevisiae* S6, a standard sensitive strain lacking M-dsRNA, on pH 4.7 medium. Killing is indicated by a clear zone of growth inhibition surrounding the patch. Each pair of panels shows the result of growth on low-phosphate (left) and high-phosphate (right) medium to test for repressibility by phosphate. (I) Control of sensitive strain S6. (J) Standard killer strain K12-1 (not repressible). (A through H) Sensitive strain transformed with eight plasmids (YGB-1 to -16) in which the preprotoxin gene of M_1-cDNA is under control of the phosphate-repressible *PHO-5* promoter (see text, "In Vivo Expression of Preprotoxin cDNAs"). All transformants are repressible killers. (Lower) Lawns of strains to be tested for immunity were exposed to patches of killer strain K12-1 (CD GH KL) or control patches of sensitive strain S6 (AB EF IJ). (A through D) Lawns of strain K12-1; (I through L) lawns of strain S6; (E through H) lawns of the transformant (YGB-16) shown in upper (H). For each set, the left pair was grown on high phosphate pH 4.7 medium, and the right pair was grown on low phosphate medium. Strain K12-1 is immune on both media, but the transformant is only immune on derepressing (low-phosphate) medium.

of autonomy. The relatively stable copy number of these dsRNAs implies some degree of coupling of VLP replication to the yeast cell cycle. Partition of VLPs during mitotic and meiotic cell division may also be controlled (5).

The *S. cerevisiae* killer system has become the best understood of the mycoviruses, primarily because of the facility of genetic and biochemical analyses in its host and the simplicity of scoring for killer toxin production and toxin immunity. Genetic analysis of this system has been extensive (reviewed in references 152, 156, 158, 160) and illustrates the complexity of nuclear-cytoplasmic and plasmid-plasmid interactions involved in maintenance and expression of the killer M-dsRNAs. Relationships observed between different ScVs or dsRNA species include dependency, indifference, and incompatibility or exclusion. The killer system is now a prime model for the investigation of such phenomena in eucaryotic microorganisms. Genetic analysis of this system continues to reveal levels of complexity that outstrip biochemical analysis of their implications, although recent advances have clarified many previously obscure issues. Description of these findings is the major rationale for writing a new review of this subject.

Maturation of preprotoxin, the product of the M_1-dsRNA toxin gene, is a useful model for the complex protein secretion pathway in yeast (see "Structure of the M_1-dsRNA Preprotoxin Gene and Steps in Toxin Maturation"). At the same time, recent data on the mode of action of the toxin suggest that toxin and immunity determinants will be useful

probes of yeast cytoplasmic membrane function and models for transmembrane ionic pore formation. It also seems likely that analysis of *MAK* and *SKI* gene functions (see "Nuclear Mutations Affecting M-dsRNA Maintenance") will yield valuable insights into the molecular biology of gene expression in yeasts.

Several excellent reviews of this field already exist, and the reader is especially directed to recent reviews by Wickner (160) and Bussey (23) for more extensive coverage of information in their areas of expertise, respectively, the genetics and physiology of the *S. cerevisiae* killer system. Older reviews by Wickner (156) and Bruenn (17, 112) are also valuable summaries of genetic and molecular aspects of the field.

VARIETIES AND PREVALENCE OF YEAST KILLERS

The killer phenotype was first described in laboratory strains of *S. cerevisiae* by Makower and Bevan (M. Makower and E. A. Bevan, Proc. Int. Congr. Genet. XI **1**:202, 1963). Killing was found to be caused by an exocellular, heat- and protease-sensitive toxin (169) that was only active and reasonably stable in the pH range of 4.2 to 4.7. This is now known to be close to its pI of 4.5 (107). Several more recent surveys of yeast strain collections and natural isolates have demonstrated the presence of similar phenomena in many genera (29, 65, 90, 92, 111, 116, 130, 170).

Most surveys have used rich agar media containing methylene blue in the pH range of 4.2 to 4.7, seeded or thinly spread with potentially sensitive tester strains and heavily streaked with potential killers. Zones of growth inhibition surrounding the latter, bounded by blue-stained dead yeast cells, indicates killer toxin secretion. Figure 1 shows two natural K1 killer strains and an artificial killer strain containing no killer dsRNA but containing, instead, a phosphate-repressible cDNA copy of such a dsRNA (see "In Vivo Expression of Preprotoxin cDNAs").

An *S. cerevisiae* strain sensitive to the type K1 *S. cerevisiae* killers has often been used for initial screening, followed by cross-testing of killers to define immunity patterns. Such cross-testing matrices (116, 130, 170) usually demonstrate that, within a given genus, each killer strain is immune to its own toxin, but sensitive to toxins of other immune classes. This simple pattern is complicated by variation in the sensitivity of nonproducing strains, or of strains which all produce a single toxin type, to other toxins. As pointed out by Young and Yagiu (170), such strain variation in sensitivity is probably a result of nuclear mutations to resistance.

It is useful to distinguish semantically between killer-specific, plasmid-determined immunity and less specific resistance related to nuclear genotype. For example, whereas intergeneric killing does occur, many yeast strains are intrinsically resistant to killer toxins produced by members of other genera. Known *S. cerevisiae* nuclear mutations (*kre1,2,3*) leading to resistance to M_1-dsRNA-determined K1 toxin also confer resistance to a broad range of other toxin types (1, 26). However, neutral strains carrying a mutant M_1-dsRNA which fails to cause toxin production (7, 24) retain immunity to the K1 toxin only.

The most thorough survey of killer types was performed by Young and Yagiu (170), using 20 killer strains found earlier in the British National Collection of Yeast Cultures (NCYC) (111). Species in 7 of 28 genera produced toxin active against an *S. cerevisiae* strain. Thirteen negative genera were represented by only one or two species, and since prevalence of killers among positive genera varied from 2 of 51 (*Candida*) to 12 of 29 (*Hansenula*), it seems likely that broader screening would turn up killers in several other genera. Exocellular toxins produced by the killers could be distinguished on the basis of pH optima, temperature stability, and relative susceptibility to proteases, although all were protease sensitive and optimally active below pH 5. Ten toxin types (K1 to K10) were distinguished by killer toxin and immunity specificities, including three (K1, K2, and K3) in *Saccharomyces* sp. strains. K1 is the type originally recognized in laboratory strains of *S. cerevisiae* (6, 7; Makower and Bevan, Proc. Int. Congr. Genet. XI **1**:202, 1963). K2 killers were first recognized in Russian wine yeast and were shown to be capable of killing K1 killers (97, 98). Similar strains were found as contaminants of a two-stage continuous beer fermentation (88) and were later shown to belong to the same K2 killer group (116). K3 is currently defined by strain 761 of *S. capensis* (170). K1, K2, and K3 killers were found to carry physically distinct species of M-dsRNA (1.9, 1.7, and 1.5 kilobases [kb], respectively) (170). Minor differences were also reported in the mobilities of their associated L-dsRNAs (170). The plasmid nature of the M_1-dsRNA determinant of the K1 killer strains had been previously indicated by demonstration that it could be cured by growth at high temperature (151) or in the presence of subinhibitory concentrations of cycloheximide (46) or 5-fluorouracil (95, 99) with concomitant loss of the killer character. This was also shown to be true for the K2 and K3 killer types (170). No dsRNA species were detected in any member of other killer classes, and none of these killers were curable under these conditions.

A homothallic wine yeast (*S. cerevisiae*) has recently been described (43) that kills both K1 and K2 *S. cerevisiae* killers. After nitrosoguanidine mutagenesis, strains killing both, called "K3," and strains bearing the K2 killer phenotype were found. The original strain contained two M-dsRNA species of about 2.0 and 1.5 kb. The K2 derivatives contained the 1.5-kb species, presumably closely related to 1.7-kb M_2-dsRNA, whereas the K3 derivatives contained the 2.0-kb species. This is apparently a new killer plasmid, compatible with M_2-dsRNA. It is considerably larger than the *S. capensis* M_3-dsRNA of Young and Yagiu (170), so it is probably a fourth type of M-dsRNA, though this has not been tested directly. If the unusual killer toxin produced by *S. cerevisiae* strain 28 (110) is associated with a unique dsRNA, this will presumably be a fifth type. This strain produces an unusually stable toxin with a pH optimum of 5, higher than for any other known killer. Its genetic determinant is unknown (110).

Killing of *S. cerevisiae* by *Torulopsis glabrata* strain ATCC 15126 was demonstrated in 1975 (29, 30). Its toxin, dubbed pool efflux-stimulating toxin (PEST), acts very much like the other yeast killer toxins (27, 77, 89) except that its action is apparently independent of energy consumption by the victim (124). The toxin specificity of this strain differs from all of those tested by Young and Yagiu (170), including their K4 strain, *T. glabrata* 388. It was called "Tox 3" by Rogers and Bevan (116), but has been appended by Wickner (156) to the Young and Yagiu classification (170) as K11 specificity.

Extensive investigations of killing in natural yeast isolates by Middelbeek et al. (89–94, 130) has almost certainly uncovered other unique killer types. However, no complete comparison of these strains with the K1 to K11 types (156) has been reported, nor has the nature of the genetic determinants or their mode of transmission been reported for any of these isolates. Among 157 natural isolates, 17%, representing seven separate genera, were killers of *S. cerevisiae* (130).

Among yeast genera commonly found as opportunistic human pathogens, K1 killers were first observed in *Candida* spp. by Mitchell et al. (Abstr. IV Int. Congr. Yeast Genet. Mol. Biol., p. 24, 1975), and K11 was seen in *Torulopsis* sp. by Bussey and Skipper (29). An extensive survey by Middelbeek et al. (91), using a wide variety of killer strains, showed sensitivity among 116 of 142 strains of *Candida* and *Torulopsis* spp., especially to toxins produced by strains of *Hansenula* sp. (130) and *Pichia kluyveri* (90). Sensitivity is now being used epidemiologically for typing of fungal pathogens (113). A much lower prevalence of sensitivity was found in a similar survey by Kandel and Stern (69). However, the latter study used only *S. cerevisiae* and *Torulopsis* sp. strains as sources of toxin. *Cryptococcus* sp. strains were found to be sensitive only to toxins produced by other cryptococci and by certain *Torulopsis* sp. strains (92).

MODE OF ACTION OF KILLER TOXIN

The mode of action of killer toxins has been studied extensively in the *S. cerevisiae* K1 system, using strain K12-1, a normal laboratory strain killer, or derivatives of strain T158c. This strain contains a mutant "superkiller" form of M_1-dsRNA encoding normal quantities of a more stable toxin (107) (see below, subsection "dsRNA Determinants"). The toxin of strain K12-1 consists of two polypeptide components (10), barely resolvable on reducing gels into slower (α) and faster (β) migrating species of about 9.5 and 9.0 kilodaltons (kd) (Fig. 2). These components are disulfide linked and run as a 17-kd dimer on nonreducing gels. It is assumed, but not proven, that this is an αβ dimer. Strain M5c contains the T158c superkiller M_1-dsRNA (143) and its α component migrates more slowly than that of K12-1 (Fig. 2), although its β component migrates identically. Sequence analysis of cDNAs (10, 125; see "In Vivo Expression of Preprotoxin cDNAs") confirms that the only amino acid variations occur in the α component. In strain M5c, secreted toxin comprises about 5% of the total secreted protein (Fig. 2) (107).

Type K1 toxin is irreversibly inactivated at pH values over 6.5, is most stable at pH values near its pI (4.5), and is inactivated with a half-life of about 60 min in culture media of normal strains (31). Degradation is inhibited by phenylmethylsulfonyl fluoride and a mutation (called *ski5*) results in loss of this phenylmethylsulfonyl fluoride-inhibitable exocellular protease and markedly enhanced toxin accumulation, equivalent to that seen in the presence of phenylmethylsulfonyl fluoride in normal strains (31). A considerable fraction of toxin, partially purified according to size, is inactive. 1,6β-D-Glucan affinity (64) and controlled-pore glycerated glass (107) columns separate active toxin (10 to 30% of the total) from inactive toxin, with essentially complete retention of activity. However, the two fractions are indistinguishable on gels (25) or by interaction with polyvalent toxin-specific antisera (11).

The α and β components of toxin have a high content of hydrophobic and charged amino acids (10), with a combined pI of 4.5 (107), and are stabilized by 15% glycerol at this pH (107). Toxin action involves an initial, rapid, energy-independent binding of toxin to a cell wall receptor (1, 26) that has now been identified as a 1,6β-D-glucan (64). Both sensitive and immune (M_1-dsRNA containing) cells have a large number of such receptors, whereas mutations in either of two nuclear loci (*kre1* and *kre2*) (1) drastically reduce cell wall binding and modify 1,6β-D-glucan content. *kre* mutants are resistant to a wide range of killer toxins, suggesting a common initial binding step in their action (1, 26, 64).

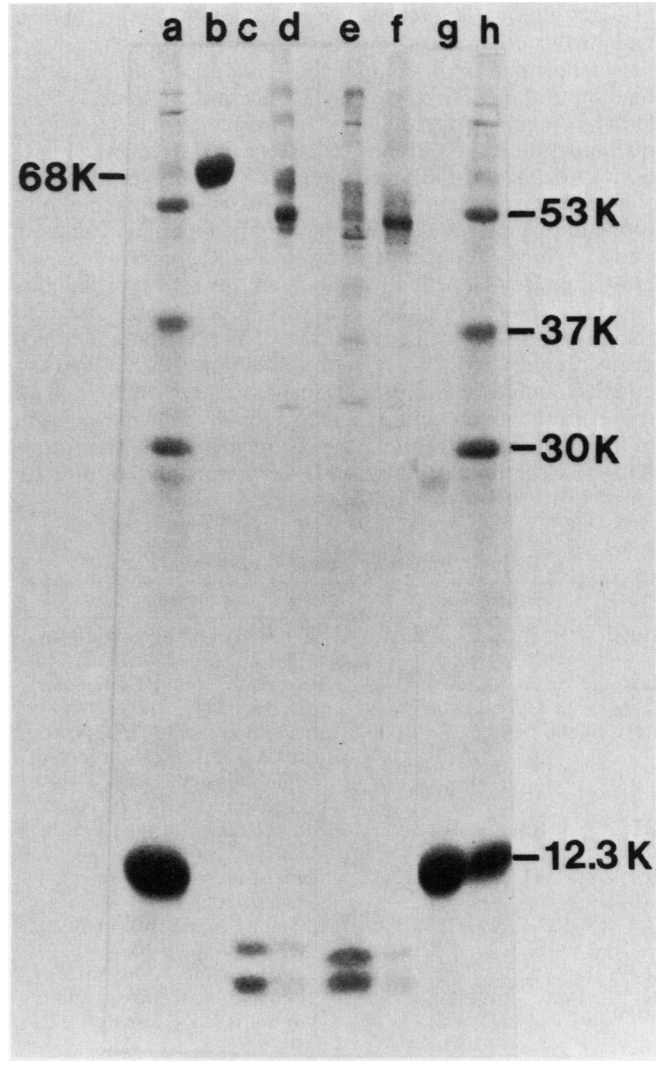

FIG. 2. Fractionation of total exocellular proteins and partially purified toxins from killer strains by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10). (Lanes a, b, g, h) Marker proteins; (lanes c, d) purified and crude toxin from strain M5c, containing the *ski5* mutation (31) and superkiller T158c M_1-dsRNA mutant, modified in the α-toxin component sequence (see text, "In Vivo Expression of Preprotoxin cDNAs"); (lanes e, f) purified and crude toxin from wild-type killer strain K12-1.

After binding to the wall, an energy-dependent process (124) transfers toxin to its site of action, the cytoplasmic membrane. As few as 3×10^4 of the 10^7 cell wall-binding sites need to be occupied for this step and lethality to occur (23). *kre1* and *kre2* mutant cells have toxin-sensitive protoplasts, whereas the protoplasts of immune, toxin-producing cells retain their immunity (28). Mutants in a third nuclear gene, *kre3* (1), are resistant in spite of normal wall binding and their protoplasts remain resistant (H. Bussey, personal communication).

The non-saturability of immunity argues against direct interaction of toxin and immunity determinants. This and the existence of the *kre3* and *rex* mutants (in which killer plasmid-carrying strains kill themselves; 150) suggest the existence of a receptor in the membrane for the second stage

of toxin action. At present it is not known whether both α and β toxin components interact with this putative receptor, or whether one toxin component is required only for initial binding to the wall, serving as a concentrated source of the lethal component for delivery to the membrane (see "Sequence of the M_1-P1 Preprotoxin Gene," subsection "Functional Analysis of the Toxin Subunit Structure and γ").

The events leading to cell death also require expenditure of metabolic energy (27). Killing is only apparent after a lag period which is dependent on medium composition and growth phase. The effects of toxin are reversible during this lag, and stationary-phase cells are relatively resistant to toxin. In growing cells, inhibition of net proton efflux (39) occurs rapidly, coincident with inhibition of K^+ influx and coupled amino acid-proton transport activity (38). All of these effects are apparently due to a rapidly induced increase in the permeability of the cytoplasmic membrane to protons (38). The proton extruding ATPase is probably not directly affected, but its effects are overwhelmed, leading, over a period of 40 to 90 min, to a marked drop in intracellular pH, metabolic inhibition, potassium ion efflux, and cell death associated with the leakage of metabolic pools such as ATP (27, 38). Macromolecules do not leak.

Very similar observations have been made with the toxin from a sake strain of *S. cerevisiae* (65, 77) which shares K1 immunity (23). It was also observed with this strain that Ca^{2+} ions prevented toxin-induced membrane damage (77).

Recent and extensive investigations of the mode of action of the purified toxin from *P. kluyveri* 1002 have extended these observations. This yeast strain kills *S. cerevisiae* K1 killers (90), although it shares most of their killing spectrum (130). Its relationship to the current killer classification and the nature of its killer determinant are unknown. Like the K12-1 toxin, the *P. kluyveri* toxin is most active and stable at pH values near its pI of 4.3. It has an apparent molecular weight of 19,000 and may be a glycoprotein (90). Binding to a cell wall receptor is energy independent, and when added to cells pretreated with metabolic inhibitors such as dinitrophenol or antimycin A, cells can be rescued simply by raising the pH to 7, inactivating cell wall-bound toxin (94). After energy-dependent insertion into the membrane, killing can still be reversed for some time, but now requires removal of toxin and adjusting medium pH and K^+ concentrations to physiological levels (pH 6.5, 50 mM KCl; 89). Cells are still killed if continuously exposed to toxin under these conditions for 60 min (89, 94) since membrane-associated toxin apparently remains active at pH 6.5, so that cumulative damage still occurs. It has recently been shown (68) that this toxin forms relatively nonselective, ion-permeable channels in artificial phospholipid bilayer membranes. Similar observations have been made with the T158c type K1 toxin (Bussey, personal communication). These observations further exemplify the resemblance between the yeast killer toxins and the E1 functional class of colicins (119).

As pointed out by Kagan (68), the conductance of killer toxin channels is approximately 100 times that of colicin E1 channels, consistent with the observation that toxin action on yeast cells, whose volume is about 1,000 times that of an *Escherichia coli* cell, like colicin action on *E. coli* cells, can cause an immediate leak large enough to inhibit net proton pumping and to dissipate preexisting membrane potential. This leak is also large enough to allow the cumulative lethal effects of leakage to occur within a cell's generation time in both *E. coli* and yeasts.

TABLE 1. Nomenclature for *S. cerevisiae* killers

Phenotype(s)	Definition
Killer	Secretion of a polypeptide toxin that kills sensitive yeast cells
Immune	Plasmid-determined resistance to homologous killer toxin
Resistance	Resistance to a broad range of toxins, dependent on growth conditions or nuclear genotype (e.g., lack of receptor)
K1, K2, or K3	Strain having the type 1, 2, or 3 killer phenotype
R^-	Lack of immunity to known killer toxins
R_1^+ R_2^+ R_3^+	Immunity to K1, K2, or K3 toxin, respectively
R_1^W	Weak immunity to K1 toxin
K_1^+	Production of K1 toxin; ability to kill R^-, R_2^+, or R_3^+ strains
K_2^+	Production of K_2 toxin: ability to kill R^-, R_1^+, or R_3^+ strains
K_3^+	Production of K3 toxin: ability to kill R^-, R_1^+, or R_2^+ strains
K_1^{++}	Superkiller producing more active or more stable K1 toxin
(K_1^+ R_1^+) (K_2^+ R_2^+)	Normal phenotypes of K1 and K2 killer strains, respectively
(K^- R_1^+)	Neutral phenotype; strain carries a mutant K1 plasmid
(K_1^+ R_1^W)	Suicidal phenotype; strain carries a mutant K1 plasmid
Exclusion	Interference with replication of a killer plasmid leading to its loss by dilution; can be a consequence of plasmid or nuclear genotype
Suppressive	Plasmid causing exclusion
Helper	Plasmid preventing exclusion or enabling replication
Satellite	Replication-defective plasmid dependent on another; e.g., ScV-M_1 is a satellite of ScV-L_{1A}
VLP	Virus-like particle
ScV-L_{1A}	*S. cerevisiae* virus containing L_{1A}-dsRNA in VLPs

SUMMARY OF PHENOTYPES, dsRNA DETERMINANTS, AND PLASMID GENOTYPES OF *S. CEREVISIAE* KILLERS

Nomenclature

The nomenclature used for killer phenotypes is summarized in Table 1. *S. cerevisiae* killers of types K1, K2, and K3 have immunity to homologous toxin (R_1^+, R_2^+, and R_3^+ phenotypes, respectively) and secrete toxins of appropriate specificity (K_1^+, K_2^+, and K_3^+, respectively).

Standard genetic tests (see below) distinguish the cytoplasmically inherited killer phenotypes determined by multicopy plasmid genomes from those phenotypes affecting killer maintenance and expression which are determined by nuclear genes subject to Mendelian segregation (*MAK*, *SKI*, etc.; see "Nuclear Mutations Affecting M-dsRNA Maintenance"). (The dominant alleles of yeast nuclear loci are capitalized [e.g., *SKI5*].)

TABLE 2. dsRNAs in *S. cerevisiae* killers

dsRNA(s)	Definition/comments
M_1	1.9-kb dsRNA; determinant of the K_1^+ R_1^+ phenotype
M_2	1.7-kb dsRNA; determinant of the K_2^+ R_2^+ phenotype
M_3	1.5-kb dsRNA; determinant of the K_3^+ R_3^+ phenotype
S3	0.73-kb dsRNA derived from M_1 by an internal deletion
L_A	4.7-kb dsRNA competent and required for maintenance of M_1 or M_2-dsRNAs; requirements for M_3 maintenance not determined
L_{1A}	4.7-kb class L_A dsRNA found in natural K1 killers; encodes an 88-kd polypeptide that encapsidates itself and M_1
L_{2A}	4.7-kb class L_A dsRNA found in naturally isolated type K2 killers; only partly homologous to L_{1A} but probably has similar functional capabilities; encodes the 84-kd capsid of its own VLPs
L_B, L_C, L_{BC}	4.7-kb dsRNAs found in most K1 and K2 killers; related to each other but not to L_{1A} or L_{2A}; not required for, nor probably capable of, maintenance of M_1 or M_2; encode the 82-kd capsid of their own VLPs
T, W	Minor 2.7- and 2.25-kb dsRNA species of unknown function present in many *S. cerevisiae* strains; unrelated to each other or to other known dsRNA species
XL	Minor dsRNA slightly larger than L_{1A}, occasionally reported in K1 killers; identity unknown

dsRNA Determinants

The dsRNAs associated with these plasmid phenotypes are listed in Table 2. The *S. cerevisiae* system is comprised of essentially two segments of dsRNA distinguished by size, L and M, which may exist in multiple subspecies. In addition, minor species designated T, W, and XL have been observed in some strains. The products of in vitro translation of these dsRNAs (after denaturation) and their presumed or proven in vivo counterparts are shown in Table 3.

Most isolates of *S. cerevisiae* contain at least one species of 4.7-kb L-dsRNA. Maintenance of an M-dsRNA is dependent on a subspecies, called L_A, in which considerable variation is found. Variants associated with naturally isolated type K1 and K2 killers are called L_{1A} and L_{2A}, respectively (Table 2; "Role of L-dsRNA Species in VLP Capsid Production and M_1-dsRNA Maintenance"). A second subspecies, L_{BC}, is often present as a minor additional component. It cannot, by itself, support M-dsRNA maintenance (Table 2).

Functional Model of M_1-dsRNA

A model of M_1-dsRNA is shown in Fig. 3. The relevant data for its derivation are described elsewhere in this review. In summary, M_1-dsRNA consists of approximately 1.0- and 0.6-kb segments separated by a highly adenine-uracil (AU)-rich denaturation "bubble" sequence, probably of variable size (here shown as 200 base pairs [bp]). The longer segment encodes the toxin precursor, preprotoxin. Preprotoxin, the primary in vivo translation product of M_1-dsRNA transcripts, is believed to be identical to M_1-P1, the product of in vitro translation of denatured M_1-dsRNA (Table 3). The two disulfide-linked toxin polypeptides, α and β, are about 9.5 and 9.0 kd, respectively (Table 3) (10). They are separated in preprotoxin by a segment called γ, predicted to be the immunity determinant, and are preceded by an N-terminal segment called δ. The site of action of nuclear gene products involved in expression (maturation of toxin from preprotoxin; *SEC*, *KEX*) and maintenance (*MAK*, *SKI*) of M-dsRNA are also shown.

Plasmid Genotypes of *S. cerevisiae* Killers

The plasmid genotypes of the M-dsRNA variants in *S. cerevisiae* killers are listed in Table 4. These are designated [KIL-k_1], [KIL-k_2], and [KIL-k_3], respectively, for M_1-, M_2-, and M_3-dsRNAs. Thus, a normal K1 killer strain contains M_1-dsRNA in ScV-M_1 VLPs and has a [KIL-k_1] genotype and a (K_1^+ R_1^+) killer phenotype. Strains carrying no M-dsRNA are sensitives (K^- R^-) with a [KIL-o] genotype usually containing an L-dsRNA.

Because the M-dsRNAs are stably maintained and segregated, mating of a sensitive, *MAK*$^+$ [KIL-o] strain with a K1 killer ([KIL-k_1] genotype) produces a K_1^+ R_1^+ diploid which, on sporulation, gives rise to 4:0 segregation of [KIL-k_1] killers (7, 127). Strains carrying a recessive *mak* nuclear

TABLE 3. dsRNA gene products

dsRNA	In vitro translation			In vivo products		
	Product name		kd	Precursor	kd	Mature
	New	Old				
L_{1A}	L_{1A}-P1	L-P1	88			VL_{1A}-P1 (ScV-P1): capsid for VLPs containing L_{1A} and M_1-dsRNAs
L_{2A}	L_{2A}-P1	L-P5	84			VL_{2A}-P1: capsid for VLPs containing L_{2A} and M-dsRNAs
L_{BC}	L_{BC}-P1	L-P2	82			VL_{BC}-P1 (ScV-P2): capsid for VLPs containing L_{BC}
	L_{BC}-P2	L-P3	78			Quite distinct peptide map from L_{BC}-P1; comigrates with ScV-P3, but identity not tested
M_1		M_1-P1	34.8 (preprotoxin)	Protoxin	42	Toxin (9.5 [α] + 9.0 [β] kd)
M_2		M_2-P1	42 (preprotoxin)	Protoxin	ND[a]	Toxin (18 kd)
M_3		ND	ND	Protoxin	ND	Toxin (ND)
T, W		ND	ND	ND		ND
S3		S3-P1	8 (contains N terminus of preprotoxin)	None detectable		None

[a] ND, Not determined.

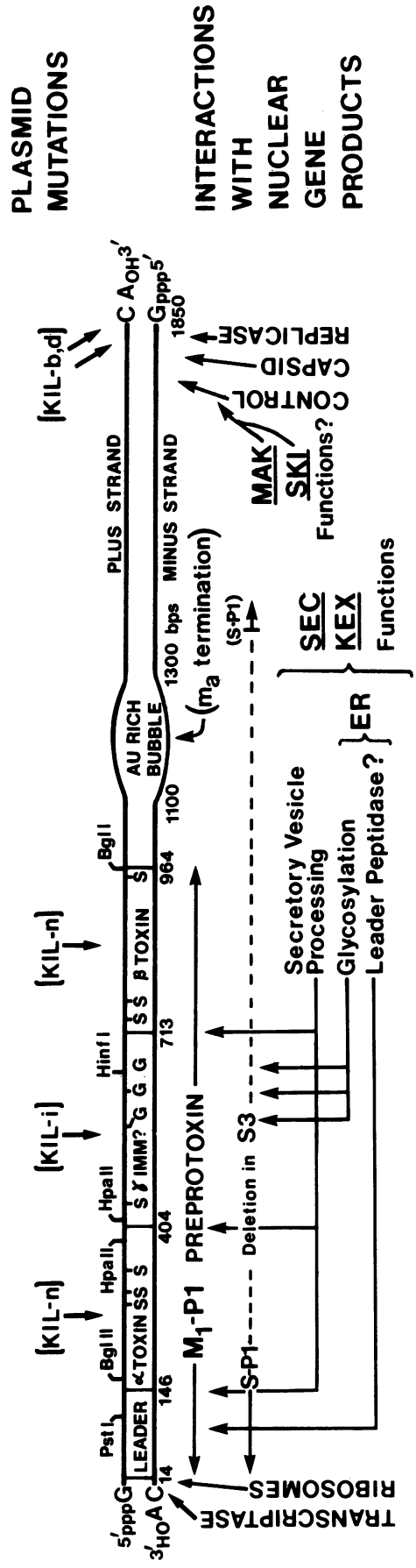

FIG. 3. Schematic structure of M_1-dsRNA. Segments encoding the β, γ, and δ components of the preportoxin gene, from bases 14 to 964 of the plus (upper) strand, are shown to scale. Cleavage sites in the cDNA covering most of this gene (heavy line, bases 111 to 1,140) are shown. The AU-rich denaturation bubble is the site of termination of the m_a partial-length transcript. ER, Endoplasmic reticulum. For other details, see text.

TABLE 4. Plasmid genotypes in *S. cerevisiae* killers

Genotype	Description	M-dsRNA	Killer and immunity phenotype
[KIL-o]	Lack known killer plasmids	None	$K^- R^-$
[KIL-k_1]	Normal K1 killer plasmid	M_1	$K_1^+ R_1^+$
[KIL-k_2]	Normal K2 killer plasmid	M_2	$K_2^+ R_2^+$
[KIL-k_3]	Normal K3 killer plasmid	M_3	$K_3^+ R_3^+$
Mutants of K1 killer plasmid			
[KIL-n_1]	Neutral	M_{n1}	$K^- R_1^+$
[KIL-i_1]	Immunodeficient (suicidal)	M_{i1}	$K_1^+ R_1^w$
[KIL-s_1]	Dominant suppressive (e.g., S3)	S	$K^- R^-$
[KIL-sk_1]	Superkiller	M_1 mutant	$K_1^{++} R_1^+$
[KIL-b_1]	Bypasses need for many *MAK* gene products	M_1 mutant	$K_1^+ R_1^+$
[KIL-d_1]	Dependent on a diploid host	M_1 mutant	$K_1^+ R_1^+$

mutation (96, 150) are unable to maintain M_1-dsRNA and so have a [KIL-o] phenotype. On mating to a [KIL-k_1] strain, a $K_1^+ R_1^+$ diploid is produced. Sporulation produces 2:2 segregation of *mak* [KIL-o] and *MAK* [KIL-k_1] strains. Recessive *ski* mutations (139) are nuclear suppressors of *mak* mutations.

Plasmid Mutations in M_1-dsRNA

Few natural mutants of ScV-M_1 have been identified (7, 24, 96, 99, 115, 126, 141, 143), possibly because of the high copy number of its dsRNA genome. Vertical transmission of this plasmid, by cytoplasmic exchange, is presumably accompanied by transfer of several copies. As a consequence, segregation appears to be slow. Moreover, selective pressure is constantly applied for sequences that are replicated and expressed at an optimal level: too low a rate will result in plasmid loss, whereas too high a rate would probably be growth inhibitory or lethal. M_1-dsRNA plasmid mutations have been defined which affect the expression of toxin activity (neutral, [KIL-n_1]), expression of immunity (suicidal, [KIL-i_1]), and M_1-dsRNA replication (*mak* bypass, [KIL-b], and diploid dependent, [KIL-d]) (Table 4). Their presumed locations are shown in Fig. 3. The [KIL-b] mutant is discussed in "Nuclear Mutations Affecting M-dsRNA Maintenance," subsection "Superkiller (*ski*) Mutants and the [KIL-b] Plasmid Mutant."

Neutral and suicidal mutants ([KIL-n_1], [KIL-i_1]). Neutral mutants of M_1-dsRNA (genotype [KIL-n_1]) conferring a $K^- R_1^+$ phenotype (Table 1) were observed early among natural isolates (7). They have a defect in the toxin precursor gene but not in the component responsible for immunity. Some neutral strains, but by no means all, secrete an inactive toxin, a cross-reactive protein (CRM) of apparently normal size that is precipitated by antitoxin (see "Effect of Plasmid Mutants on Toxin Maturation") (24). This presumably arises from a missense mutation in the toxin structural gene (Fig. 3).

A superkiller mutant strain (T158c) with a $K_1^{++} R_1^+$ phenotype (Table 1) was isolated (143) and later shown to produce presumably normal quantities of a more stable toxin (107) with a modified α component (Fig. 2).

Plasmid mutants have been isolated recently by growth of a strain carrying the M_1-dsRNA derived from T158c at 37°C, causing gradual curing of the M_1-dsRNA plasmid (24). Ethylmethanesulfonate mutagenesis was applied at a stage when a maximal fraction of cells contained a single M_1-dsRNA molecule. Cells regenerated a normal copy number when returned to 30°C and were screened for mutant killer phenotypes. A diploid strain was used to avoid detection of recessive nuclear mutations, and cytoplasmic inheritance of the phenotype was confirmed by standard tests (7). A variety of neutral (NLP) and "suicidal" (SLP) mutants were isolated.

The suicidal mutants ([KIL-i_1] genotype) produce wild-type quantities of normal toxin, but have a much reduced immunity. These are thought to be defective in the immunity determinant encoded by M_1-dsRNA (Fig. 3). They still survive, even when grown at pH 4.7 (the optimal pH for toxin stability and activity), but unlike normal killers, which survive high toxin concentrations, SLP mutants were killed by concentrations only about 100-fold greater than those required to kill sensitives derived by complete heat curing of the killer plasmid from the parent cell line (24).

Suppressive mutants ([KIL-s_1]). Suppressive deletion mutants of M_1-dsRNA (genotype [KIL-s_1]), called S-dsRNAs, were reported in 1973 (126) and shown, by heteroduplex analysis, to result from internal deletions (47, 70). Such deleted plasmids confer neither toxin production nor immunity, but on mating of a strain carrying a [KIL-s_1] plasmid to a [KIL-k_1] strain, an unstable ($K_1^+ R_1^+$) diploid is produced. It slowly segregates $K^- R^-$ [KIL-s_1] suppressives. If the unstable diploid is immediately sporulated, killer haploids produced also segregate suppressives, 4:0. The mutant plasmid excludes the normal M_1-dsRNA plasmid, giving suppressive strains a dominant ($K^- R^-$) phenotype, much as defective-interfering virus mutants interfere with replication of wild-type viruses (63). However, unlike viral defective-interfering particles, S-dsRNAs are not dependent on the parent M_1-dsRNA; rather, both M_1- and S-dsRNAs are defective satellites dependent on L_A-dsRNA for encapsidation, so that M_1 can be completely eliminated and still allow maintenance of an S-dsRNA. Since L-dsRNAs produce capsid proteins essential for their own maintenance (see "Role of L-dsRNA Species in VLP Capsid Production and M_1-dsRNA Maintenance"), one might expect to find L deletions which are the equivalent of classical viral defective-interfering particles, but which are not suppressives. Such L deletions have not, however, been observed.

In a recent study (115), it was shown that suppression is not a consequence of growth advantage of cells containing the S-dsRNA, nor of a higher copy number of S- than M_1-dsRNA. Rather, as anticipated, it seems to be due to preferential replication of the S-dsRNA, possibly because of competition for a limiting supply of some essential cellular component. Three S-dsRNAs derived from the same M_1-dsRNA were found to fail to coexist stably. The most suppressive was actually the largest of the three, so that reduced genome length is not the cause of suppression. Neutral mutants of unaltered size found coexisting with the parental M_1-dsRNA in one unstable K1 killer strain (13) were themselves suppressive (J. Sturgeon, K. A. Bostian, and D. J. Tipper, Abstr. IX Int. Congr. Yeast Genet. Mol. Biol., p. 102, 1978). This suggests that relatively subtle mutations can lead to altered dsRNA replication efficiency. This may explain the origin of natural isolates containing a single mutant type of M-dsRNA, in spite of a presumed initial excess of normal M_1-dsRNA.

The best-characterized S-dsRNA is S3, derived from the mutant M_1-dsRNA of superkiller strain T158c by an internal 1.2-kb deletion; 500- and 230-bp terminal fragments are left

(47). Sequence analysis (D. J. Thiele, E. M. Hannig, and M. J. Leibowitz, Virology, in press) has recently located these fragments in M_1-dsDRNA (Fig. 3). Two modified forms of S3 segregated during replication: a dimeric molecule (S1) and a form (S4) derived from S1 by a second small deletion (47). Similar duplication and size variation have been seen in other suppressives (115) and presumably arise by some copy choice mechanism during transcription within the VLPs.

Diploid-dependent mutant ([KIL-d_1]). A [KIL-d] mutant has been isolated in which the M_1-dsRNA is only maintained in **a**/α diploid cells (153). Maintenance of this dsRNA has apparently become subject to *MAT* locus controls.

STRUCTURAL COMPARISONS OF KILLER dsRNAs

In spite of early reports to the contrary (reviewed in reference 156), and in keeping with all of the genetic evidence, recent hybridization experiments have failed to find any homology between any killer dsRNA species and any DNA genome in *S. cerevisiae* (8, 9, 57, 129). Probes used in Southern blots of genomic DNA have included denatured and fragmented dsRNAs, labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, representing all of the L_{1A}-, L_{BC}-, and M_1-dsRNAs (9) and nick-translated cDNA complementary to 50% of M_1-dsRNA (9). Use of these probes and nick-translated cDNAs complementary to segments at one terminus of L_{1A}-dsRNA (8) also demonstrate the lack of detectable homology between M_1 and any of the L-dsRNAs and between L_A and L_{BC}.

Except for their single unpaired 3′ bases, each dsRNA is a perfect duplex. M_1-dsRNA has an internal, asymmetrically located, highly AU-rich bubble sequence of about 200 bp, visible as a denatured region in electron micrographs (47) and removed by treatment with RNase S1, leaving terminal fragments of about 1,000 and 600 bp (Fig. 3) (145). No similar region has been detected among L-dsRNAs. This difference is believed to account for the fact that, whereas L-dsRNA size is stable, M_1-dsRNA size varies by up to 12% in subclones of K1 killer strains (S. S. Sommer and R. Wickner, personal communication). The transcriptase may slip or skip while transcribing oligouridylic acid regions on the negative strand in the bubble, leading to variation in bubble size and insertion or deletion of oligoadenylic acid [oligo(A)] sequences in the plus strand.

Each dsRNA has a relatively AU-rich terminus, the source of a large RNase T_1 oligonucleotide, and a relatively guanine-cytosine-rich terminus (15). The AU-rich 5′-terminal sequences reported for L_{1A}, L_{2A}, M_1, M_2, and L_{BC}-dsRNAs are shown in Table 5.

Sequencing of dsRNAs from their 3′ termini has provided data for 100 to 250 bp of both ends of M_1- and L_{1A}-dsRNAs (133–135; Thiele et al., in press). Earlier reports of sequence heterogeneity in L-dsRNA (19) were, at least in part, due to the presence of a mixture of L_{1A} and L_{BC} and probably also to the presence in dsRNA preparations of multiple small RNA species of about 100 bp (L. A. Weinstein and M. J. Leibowitz, personal communication). These appear to be specifically associated with the dsRNAs, but are not related to the partial-length "pause products" synthesized by VLP transcriptase (14) (see "Transcription and Replication of Killer dsRNAs"). Some heterogeneity has been found in the sequences of overlapping cDNAs, representing fragments of a single L-dsRNA preparation (8; J. Bruenn; personal communication), suggesting rapid sequence divergence. In contrast, very little divergence has been seen in comparing sequences coding for toxin precursors in M_1-dsRNAs from normal and superkiller strains (see "Sequence of the M_1-P1 Preprotoxin Gene" and "In Vivo Expression of Preprotoxin cDNAs").

CELL CYCLE STUDIES OF dsRNA REPLICATION IN *S. CEREVISIAE*

Replication of the killer dsRNAs probably occurs in two distinct steps, as in reoviruses (122): a plus-strand transcript, defined as encoding the known dsRNA gene products (VLP capsid and toxin precursor), is produced in vitro by a VLP-associated transcriptase (see "Transcription and Replication of Killer dsRNAs") (14, 18, 59, 144). It must initiate at the AU-rich dsRNA terminus (see Fig. 3). It presumably acts as a template for a replicase which regenerates dsRNA by synthesis of the minus strand. Encapsidation of the dsRNA product would regenerate a VLP.

Both 5′ ends of all L- and M-dsRNAs are pppG (20), presumably the acceptor for polymerization of each strand. All 3′ ends are -CA-OH, with C pairing with pppG and the A being unpaired (Fig. 3; Table 5) (19), with the exception of L_{BC}, in which both -CA-OH and -CG-OH 3′ termini have been found (44). This minor L-dsRNA species resembles the *Ustilago* sp. dsRNAs in this respect (see "dsRNA Killer System of *U. maydis*").

TABLE 5. Terminal sequences of dsRNAs[a]

dsRNA	"Plus" genomic strand
L_{1A}	pppGAAAAAUUUUUAAAUUCAUAUAACUCCCCAUGCUAAGA. . . . . .UAGGGAAUUACCCAUAUGCA (OH)
L_{2A}	ppp GAAUAAUUUGAAUAUUC. . . . . . . .GCUUAUACACAUAUGCA (OH)
L_{BC}	ppp GAAUUUUUCCACUCGCACAC. . .
M_1	pppGAAAAAUAAAGAAAUG. . . (HO)-A-CUUUUUAUUUCUUUAC. . . . . .UAAUGCAACAGCAUAGAAGAAACACACAUC-A(OH) . . . .GUGUAGppp
M_2	pppGAAAAAUAAAGAG. . . . . . . .UUUACCUAGCA(OH)

[a] In each case sequence is shown for the plus genomic strand. The complementary sequence of the minus genomic strand is shown only for M_1, to illustrate the 3′-unpaired A(OH) residues. The AUGs followed by open reading frames are underlined in the M_1 and L_{1A} sequences.

Several experiments demonstrate that ScV replication occurs continuously throughout the yeast cell cycle (100), although Zakian et al. report a pause in ScV-L replication during the S phase (171). Our own unpublished data indicate continuous VLP capsid, ScV-L, and ScV-M_1 synthesis in pulse-labeled cells, in balanced growth, fractionated on sucrose gradients. The stability of ScV maintenance and the relative stability but wide strain variation in ScV copy number remain unexplained. In any given strain, nutrient conditions can cause ScV-L copy number to vary as much as threefold; higher numbers are found in ethanol-grown cells than in glucose-grown cells (105), and starvation for nitrogen source causes extensive degradation of preexisting L-dsRNA (33). The return of ScV-L_{1A} and ScV-M_1 to a normal copy number at 25 to 30°C, after partial heat curing by growth at 37 to 39°C (24), suggests feedback regulation of dsRNA copy number or recovery of some temperature-sensitive, rate-limiting step in VLP replication.

It has recently been shown (R. A. Sclafani and W. Fangman, personal communication) that full-length l and m transcripts of the killer L- and M-dsRNAs (see "Transcription and Replication of Killer dsRNAs") accumulate 50- to 100-fold in cells blocked at the G1/S cell cycle boundary by the use of α-factor and *cdc7* blocks. Entry into S after relief of the block leads to reduction in l, possibly due to replication, but further increase in m. Replication of dsRNA thus appears to require some cellular components involved in the initiation of DNA synthesis. These must be constitutively present to allow dsRNA synthesis in G1. They are either inactivated or selectively occupied by DNA synthesis on entry into S phase.

TRANSCRIPTION AND REPLICATION OF KILLER dsRNAs

Isolated intact VLPs containing both L- and M_1-dsRNAs also contain a transcriptase activity which, in the presence of nucleotide triphosphates, produces full-length, non-complementary, single-stranded transcripts having messenger activity for the known dsRNA gene products (14, 15, 18, 59, 144, 146). The mechanism of transcription appears to be conservative (100; Sclafani and Fangman, Mol. Cell. Biol., in press), with extrusion of the newly synthesized plus strand. However, this is difficult to establish with certainty.

The 4.7-kb in vitro, single-stranded l transcript from ScV-L_{1A} encodes L_{1A}-P1 capsid (Table 3), whereas the 1.9-kb m transcript from ScV-M_1 encodes M_1-P1, the preprotoxin. Both 5′ and 3′ sequencing of the transcript from VLPs containing M_1-dsRNA have been performed (54). The transcript is a faithful copy of the genomic dsRNA, initiating and terminating at the ends, without polyadenylation. Addition of an extra 3′-terminal purine occurs in vitro, 53% A and 38% G, although only A has been identified in vivo (19).

The strands of M_1- and L_{1A}-dsRNAs have been separated, and the minus strands have been identified by hybridization with the in vitro plus-strand transcripts (133, 134). Determination of their 3′ sequences confirms the 5′ sequences of the in vitro transcripts, which initiate at the AU-rich terminus of both L_{1A}- and M_1-dsRNAs (Table 5). This is also the natural terminus present in the larger (1 kb) S1 nuclease fragment of M_1 (145), demonstrating that the M_1-P1 gene in this fragment is transcribed toward the bubble (Fig. 3).

Full-length, plus-stranded transcripts of the killer dsRNAs are also found in vivo (9), together with unique partial-length transcripts. These partial-length transcripts, l_a (2.3 kb) derived from L_{1A} and m_a (1.2 kb) derived from M_1, share the mRNA activities of their full-length counterparts (9). Hybridization to the separated plus and minus strands of M_1-dsRNA confirms the positive polarity of both full-length and partial transcripts (54). It is not known whether any of these transcripts are capped. The ratios of these dsRNAs and their transcripts in killer strain K12-1 are shown in Table 6. Transcripts of the L_{BC} species, present as a minor L component in strain K12-1, were not investigated (9).

The existence of both full-length and partial-length plus-strand transcripts for both dsRNA species investigated suggests differential function for these two species. It seems plausible that the full-length transcripts are intermediates in a reovirus-like replication mechanism (122), whereas the partial-length transcripts, which are just long enough to encode the preprotoxin and capsid gene products, are responsible for expression of the dsRNA genes in vivo. Perhaps secondary structure or binding of capsid or other proteins masks the mRNA activities of m and l in vivo, avoiding the potential collision of ribosomes and replication machinery converging from opposite ends of a single plus strand. Alternatively, the partial-length mRNAs may not express all of the dsRNA genes: if secondary structure or bound proteins mask the known initiator AUG in the full-length transcripts, they might serve as messengers for different (unidentified) gene products derived from downstream initiation or ribosome binding sites, absent or inaccessible in the partial-length transcripts. The VLP-associated transcriptase and the immunity determinant are candidates for such unidentified products. However, immunity probably forms part of the preprotoxin (see "In Vivo Expression of Preprotoxin cDNAs").

The AU-rich 3′ termini of L_{1A}-, L_{2A}-, M_1-, and M_2-dsRNAs (Table 5) share a common eight-base sequence, AUU(U/A)UUCA-OH, postulated to be the transcriptase recognition site (15). The sequence in L_{BC}, AAAAAUUC(A/G)-OH, differs considerably (Table 5). Since the M_1-P1 gene initiates only 6 bp upstream of this site (see below), the 1.2-kb m_a transcript must initiate at the same terminus as the full-length transcript. It probably terminates within the AU-rich bubble sequence of M_1, just beyond the end of the M_1-P1 gene (Fig. 3). It is not known whether m_a is a product of premature transcription termination or of cleavage of m. Cleavage, as in the processing of some mitochondrial transcripts, might be dependent on translation initiation.

Both m and m_a bind tightly to polyuridylic acid [poly(U)]-Sepharose, suggesting polyadenylation (9). However, it has recently been shown (54) that the separated plus strand of M_1-dsRNA and the in vitro synthesized m plus strand, neither of which bears a 3′-terminal polyadenylic acid

TABLE 6. Relative molar concentrations of dsRNAs and their transcripts in K_1 killer strain K12-1[a]

Species	kb	Relative concn	Molecules per cell
L_{1A}-dsRNA	4.7	100	30
L_{BC}-dsRNA	4.7	10	3
l transcript of L_{1A}	4.7	2	0.6
l_a transcript of L_{1A}	2.3	9	3
M_1-dsRNA	1.9	69 (100)	21
m transcript of M_1	1.9	9 (14)	3
m_a transcript of M_1	1.2	11 (17)	3

[a] L_{1A}-dsRNA represents 0.03% of the total RNA in these cells, giving a copy number of about 30. The L_{BC} relative concentration is estimated from stained gels. All other relative concentrations are derived from Northern blots (9).

[poly(A)] sequence, both bind to oligodeoxythymidylic acid [oligo(dT)]-cellulose and have precisely the same thermal elution properties on poly(U)-Sepharose as the m and m_a in vivo transcripts (9). In contrast, the minus M_1 genomic strand and both strands of S3 dsRNA (deleted for the bubble; Fig. 3) fail to bind to oligo(dT)-cellulose. Binding is, therefore, due to the presence of oligo(A) regions in the M_1 plus strand, almost certainly within the AU-rich bubble sequence. Thus, the in vivo m_a transcript probably terminates after this A-rich bubble sequence, which may perform the functions usually provided by the post-transcriptionally added 3'-oligo(A) regions of yeast nuclear mRNA transcripts. The genomic RNAs of the single-stranded polioviruses and alphaviruses both possess long 3'-terminal poly(A) tracts (118).

The site of the L_{1A}-P1 capsid gene in L_{1A} is not known. However, an open reading frame does start with AUG at bases 30 to 32, continuing through the known 113-bp sequence (133). Since translation from yeast transcripts normally initiates at the first AUG, this may be the start of the capsid gene unless its context (79) (it is preceded by CCC, not a favored sequence) makes it considerably less favorable than the next downstream AUG (F. Sherman, personal communication). No other AUG exists within the 113-bp sequence and, in common with M_1- and reovirus dsRNAs (36), it seems likely that translation initiates close to the terminal transcription initiation site, probably at this first AUG. If so, then l_a, which is just long enough to encode L_{1A}-P1, probably resembles m_a in being a 5' fragment of the full-length transcript (9). It should terminate halfway through L_{1A}. The function of the 3'-terminal 2.5 kb of L-dsRNA is unknown. The stability of L-dsRNA size suggests an essential structural or genetic role.

It has been noted (82) that the m plus strand has a plausible ribosome binding site just upstream of the M_1-P1 initiation AUG codon (Fig. 3) capable of significant base pairing to yeast 16S and 5.8S rRNAs. This is believed to have little if any significance for eucaryotic nuclear transcripts where interactions with the cap and cap binding protein may be of primary importance in translation initiation. It might be significant for the cytoplasmic dsRNA transcripts, if they are uncapped; however, this is only speculation at this point. Moreover, the L_{1A} sequence lacks an obvious ribosome binding site (133). Although this might explain why denatured M_1-dsRNA is a far better in vitro messenger than denatured L_{1A}-dsRNA (the dsRNAs are uncapped), this may, alternatively, reflect the different contexts of the initiator AUG sequences. Both the short 5' sequence in M preceding the initiation AUG and that in the L_{1A} plus strand (133) are A rich and G poor, characteristics of many yeast mRNAs (60).

The full-length in vivo transcript l, but not l_a, binds to poly(U)-Sepharose (9). No AU-rich sequence is known to exist in L_{1A}, and Hannig et al. (54) found no binding of the L_{1A} in vitro transcript to oligo(dT)-cellulose, so that l, the in vivo equivalent, may be polyadenylated. However, an oligo(dT)-primed, 4.3-kb cDNA has been made from denatured L-dsRNA (16). Also, Haylock and Bevan (56) failed to find binding of the full-length l in vivo transcript to oligo(dT)-cellulose. Possibly differential binding to poly(U)-Sepharose, which requires shorter oligo(A) stretches than binding to oligo(dT)-cellulose, indicates the presence of relatively short oligo(A) stretches within the L_{1A} plus strand, perhaps terminating l_a and the L_{1A}-P1 gene. It was suggested (9) that post-transcriptional polyadenylation may be common to the presumptive in vivo replication intermediates m and l and that subsequent removal of poly(A) might leave the terminal unpaired -A-OH on the dsRNAs. However, there is no precedent for such a mechanism. Poly(A) addition now seems unlikely, and the existence of terminal -CG-OH on a major fraction of L_{BC} molecules (44) argues against a poly(A) origin for terminal -CA-OH. Perhaps post-transcriptional addition of a single 3'-terminal A or G occurs as in *E. coli* SPβ RNA phage (2).

Yeast cells appear to lack significant pools of dsRNA or ScV-P1 capsid except in VLPs (13). Production of either may, therefore, limit the rate of VLP replication. Strain K12-1 contains only about 30 molecules of L- and 20 of M_1-dsRNA per cell (Table 6). The copy numbers of full-length transcripts, averaging about 0.6 of l and 3 of m per cell in this strain (Table 6), should depend on ScV copy number, transcription rate, transcript stability, and the rate of initiation of dsRNA replication on these transcripts. The existence of these small pools of l and m suggests either that these transcripts are sequestered in polysomes or that initiation of negative-strand synthesis is rate limiting for dsRNA replication.

Replication of dsRNAs on their plus-strand transcripts initiates at their C-rich 3' termini. There is little sequence homology among dsRNAs at this site (Table 5), and Brennan et al. (15) have suggested that a short 3' stem-and-loop structure, present just before a terminal AUGCA-OH sequence, may be the replicase recognition site. In plant viral genomic RNAs, such as alfalfa mosaic virus, such a structure serves to bind capsid as a necessary part of a replication complex (78). If this is also true of the ScV-dsRNAs, then, as suggested by Brennan et al. (15), capsid production, potentially controlled by l_a concentration, could control initiation of dsRNA replication as well as encapsidation. It seems likely that capsid binding to nascent dsRNA and encapsidation would at least accompany replication, protecting dsRNA from RNase activity and regenerating a VLP. A dsRNA-specific nuclease has recently been identified in yeasts (D. J. Mead, and S. G. Oliver, Proc. XI Int. Conf. Yeast Genet. Mol. Biol., p. 46, 1982). Thus, the rate of production of the l_a partial transcript, and the efficiency of its interaction with ribosomes, may ultimately control the ScV copy number. For ScV-L_A, copy number varies from 30 to 50, in strains such as K12-1, to several thousand in the atypical sensitive strain S7 (17). As suggested by Field et al. (44), competition for a limited capsid supply among different dsRNA species might account for the reduction in copy numbers seen in mixtures of compatible species such as L_{1A} and M_1 (S. G. Ball, C. Tirtiaux, and R. B. Wickner, Genetics, in press) as well as exclusion among apparently incompatible species such as M_1 and its suppressive derivatives (see "Summary of Phenotypes . . .," subsection "Plasmid Mutations in M_1-dsRNA") and M_1 and M_2 (see "[HOK], [EXL], and [NEX] . . .," subsection "Exclusion of M_2- by M_1-dsRNA"). No in vitro model of second-strand synthesis currently exists for testing the role of capsid in this process.

ROLE OF L-dsRNA SPECIES IN VLP CAPSID PRODUCTION AND M_1-dsRNA MAINTENANCE

All K1, K2, or K3 killers contain at least one species of L-dsRNA (about 4.7 kb; Table 2) as well as the M-dsRNA killer determinant. This is also true of cells maintaining the various mutant forms of M_1-dsRNA. In fact, almost all *S. cerevisiae* isolates contain a form of ScV-L, even though a majority are sensitives lacking M-dsRNA.

The first successful attempt at using in vitro translation of denatured dsRNA to determine gene function demonstrated

that the (major) L-dsRNA of sensitive strain 3/A1 codes for the capsid protein, ScV-P1 (88 kd), which comprises at least 95% of the VLP protein in this strain (62). Antisera raised against ScV-L were shown to precipitate both ScV-L and ScV-M_1 (55), and it was later shown, in the K_1 killer strain 2-1, that ScV-P1 capsid comprised at least 95% of the protein in both separated ScV-L and ScV-M_1 VLPs (13). Minor proteins of unknown function, called ScV-P2 (82 kd) and ScV-P3 (78 kd), were consistently found in VLPs of strain 3/A1 (62) and in both ScV-L and ScV-M_1 in strain 2-1 (13). It was proposed that encapsidation is essential for dsRNA maintenance and that M_1 is a satellite "mycovirus" dependent on L as a source of VLP capsid (62). Reciprocal dependence of L on M_1 clearly does not exist, so that M_1 and L are not parts of a single mycovirus system.

Nomenclature and Function of the Various L-dsRNA Species

It has now been shown that most *S. cerevisiae* strains contain at least two unrelated L-dsRNA species, as first suggested by Sommer and Wickner (128). The M-dsRNA killer plasmids are dependent satellites of one L-dsRNA class, whereas a second L-dsRNA class is unrelated to the first or to M. A third class of dsRNA molecules, called T and W, has recently been described (see "T-, W-, and XL-dsRNAs") and others may exist. Laboratory yeast strains are known which have most combinations of these three potentially independent ScV types, including abscence of any detectable dsRNA. Their role, if any, in cell metabolism is, therefore, obscure. This is discussed further below ("T-, W-, and XL-dsRNAs"), following descriptions of the structural relatedness, gene contents, dependence on nuclear genes, and relationship to M-dsRNA maintenance of the different L-dsRNA species.

The L-dsRNAs found in K1 killers and related strains were called L_1 and L_a by Field et al. (44), L_A, L_B, and L_C by Sommer and Wickner (129), and "L_2" and "L_1" by El Sherbeini et al. (41) (L_1 = L_A = "L_2"; L_a = L_B or L_C = "L_1"). "L_1" in native form migrates slightly more slowly than "L_2" on agarose gels (41). Whereas this difference has been seen reproducibly, it is only obvious after application of a rapid isolation procedure (41) and might reflect a modification specific to "L_1" (e.g., bound protein) rather than true size difference. In general, all recognized L-dsRNAs are of approximately the same size (estimated at 4.5 to 5.0 kb). Field et al. (44) called the major L species in K2 killers L_2, since they found it to differ from L_1 in terminal sequence (15) (Table 5), and noted that their K2 killers also contained L_a.

Nomenclature in the L-dsRNAs needs clarification. Because numbers have already been adopted to designate the K1, K2, etc., killer phenotypes, any numbers associated with L-dsRNAs should reflect a relationship to the M_1-, M_2 (etc.)-dsRNA killer determinants. The L_A, L_{BC} nomenclature of Sommer and Wickner (129), adopted here, seems the most useful primary distinguishing system. L_B and L_C have been distinguished in four strains (129) and appear to be closely related. The similar species found in other strains will be called L_{BC}. Variants of L_A are all competent, to a variable extent, for the maintenance of M_1- and M_2-dsRNAs, whereas variants of L_{BC} are not. Evidence is accumulating to indicate that this reflects the ability of capsid encoded by L_A to encapsidate M-dsRNA in VLPs. Capsid encoded by L_{BC} is known only to encapsidate itself (see below).

Although the known variation in L_{BC} properties is small, a broad spectrum of L_A phenotypes is already known. The variants found in naturally isolated K1 killer strains share only partial sequence homology with those of K2 strains studied and encode distinctly different capsid proteins. We shall call the species isolated from killer strains L_{1A} and L_{2A} to denote the associated killer type, after Bruenn (44), although it appears probable that they are quite similar in their abilities to maintain M_1- and M_2-dsRNAs. L_A subspecies vary in their ability to maintain M_1 and M_2 dsRNAs in different nuclear and cytoplasmic genetic backgrounds {*SKI*$^+$ or *ski*$^-$, etc.; see next section, subsection "Superkiller (*ski*) Mutants and the [KIL-b] Plasmid Mutant"}. The associated [HOK], [NEX], and [EXL] phenotypes (see below) have been denoted $L_{A\text{-}HN}$, $L_{A\text{-}E}$, etc. (129). We shall use this nomenclature where natural association with M-dsRNA is not known, as for $L_{A\text{-}E}$ from the sensitive strain AN33 (129). The L_A type found in all naturally isolated K1 killers analyzed is $L_{A\text{-}HN}$, which we shall call $L_{1A\text{-}HN}$.

The potentially independent VLP systems which include these different L-dsRNAs are called ScV-L_A (including ScV-L_1A and ScV-L_{2A}) and ScV-L_{BC} (Table 3). ScV-L_{BC} is apparently found in both K1 and K2 killers (44; our unpublished data) (Table 2), so it must be compatible with L_{1A} plus M_1- or L_{2A} plus M_2-dsRNAs.

Comparison of Sequences of L-dsRNAs

Apart from the apparent slight difference in size between L_{BC} and L_A detected on agarose gels (41), L-dsRNAs appear to be remarkably constant in size even when isolated from *S. cerevisiae* strains of diverse origin. It has recently been shown (133) that the separated, single strands of L_{1A} are easily resolved from those of L_B or L_C on native 5% acrylamide gels, presumably as a consequence of different secondary structures. The strands of L_B, however, were not clearly resolved from those of L_C by this procedure.

In most K1 killers studied, L_{1A} comprises 90 to 95% of the L-dsRNA and L_{BC}, only 5 to 10%. However, occasional K1 strains are found in which L_{BC} is a considerably larger fraction of the total. Such strains (e.g., K23) are inefficient killers with low L_{1A} and M_1 content (our unpublished data). Three killer strains (K7, K22, and K12) and one sensitive strain (S7) are now known to contain only L_{1A} (41, 129; our unpublished data). These strains have a higher content of L_A-dsRNA than strains also containing L_{BC}. A reciprocal relationship between L_A and L_{BC} copy number is suggested. Appropriate isogenic strains must be studied to test this possibility. A decrease in L_A content probably also reduces the M_1 content of K1 killers, because of the dependency of M_1 on L_A. Conversely, nuclear *ski* mutations leading to a selectively higher M_1 content also depress L_A copy number (see "Effect of *mak* and *ski* Mutations on L-dsRNA Maintenance").

The K2 killer strain Y110 contains only L_{2A} and M_2 (our unpublished data), whereas K2 killer strain 482 also contains L_{BC}. Thus, L_{BC} seems to play no necessary role in maintenance of M_1-, M_2-, L_{1A}-, or L_{2A}-dsRNAs. Formally, L_{BC} belongs to a separate mycovirus, ScV-L_{BC}, whose interaction with ScV-L_A and ScV-M species is largely characterized by indifference.

Terminal sequencing of these dsRNAs is progressing (15, 19, 44, 133) (Table 5) and shows little conservation beyond the first nine bases. Most of the available information on internal sequence relatedness among dsRNAs depends on hybridization analyses and T_1 nucleotide maps. These nucleotide maps show no relationship between L_{1A} and L_B or L_C and only slight homology between L_B and L_C (129). Liquid hybridization also shows L_{1A} to be unrelated to L_B or L_C; however, L_B and L_C are about 50% related by such an assay

(129). It is calculated that as little as 12% random sequence modification is required to give totally different T_1 fingerprints (129). "Northern" blot analyses confirm these findings. Portions of L_{1A} (200 to 400 bp) have been cloned as cDNAs, using short in vitro transcription products derived from the 5′ terminus as template (8). A cDNA probe representing 325 bp close to the AU-rich terminus of L_{1A} and a second probe derived from a region near this terminus, but separated from it by at least 585 bp, failed to react with L_{BC} (8). Use of labeled fragments representing all of L_{1A} and L_{BC} as probes also confirms the lack of detectable homology between L_{1A} and L_B or L_C, and between L_{BC} and L_{1A} or L_{2A}, and confirms the marked similarity of L_B and L_C (137; our unpublished data). The L_{1A} probe cross-hybridized about 20 to 30% with L_{2A} (our unpublished data). The terminal cDNA probe derived from L_{1A} also reacted moderately strongly with L_{2A}, whereas the internal cDNA probe failed to react (8). Only limited regions of L_{1A} and L_{2A} may, therefore, be sufficiently homologous in sequence to cross-hybridize. L_{1A} and L_{2A} are distinctly less related than L_B is to L_C.

Capsid Proteins Encoded by the Various L-dsRNAs

The gene products presumed or proven to be encoded by various L-dsRNAs are listed in Table 3. Translation of denatured L_{1A} produces L_{1A}-P1, the previously identified 88-kd major ScV-P1 capsid in K1 killers (62), which is capable of encapsidating M_1-dsRNA (13). This is now called VL_{1A}-P1 (Table 3).

Translation of L_{2A} produces L_{2A}-P1 (84 kd; Table 3), identical to the VL_{2A}-P1 major capsid protein in strain 482 killer VLPs. L_{2A}-P1 is quite distinct from L_{1A}-P1 in its peptide map (34; M. El Sherbeini et al., submitted for publication).

Translation of denatured L_B, L_C, or L_{BC} produces L_{BC}-P1, an 82-kd polypeptide that has been shown, by peptide mapping (34), to be identical to the single capsid protein in VLPs containing L_{BC} (VL_{BC}-P1, Table 3) (El Sherbeini et al., submitted for publication). VLP capsid proteins from strains containing only L_{BC} were previously reported (129) to comprise a mixture of components smaller than L_{1A}-P1. However, L_{BC}-P1 is extremely sensitive to proteolysis and the lower-molecular-weight species may be degradation products. Peptide maps fail to distinguish between the 82-kd capsids from VLPs isolated from strains containing only L_B, only L_C, or L_{BC}-dsRNAs. However, these capsids are clearly different from those of VLPs containing L_{1A}- and L_{2A}-dsRNAs (El Sherbeini et al., submitted for publication).

L_{BC}-P1 was previously called L-P2, a major translation product of denatured L-dsRNA from strains such as K12-1 which contain both L_{1A} and L_{BC}. Even though L_{BC} is only 5 to 10% of the dsRNA in these strains, it is apparently a much more efficient in vitro messenger and L-P2 (L_{BC}-P1) is often the predominant translation product. VL_{BC}-P1 comigrates with ScV-P2, first identified as a minor VLP component in strain 3/A1, a strain containing L_{BC} (62; our unpublished data). However, ScV-P2 is also seen in strain 2-1, which contains only L_{1A} (our unpublished data), where its source is unknown. The source of ScV-P3, seen in VLPs of both strains 3/A1 (62) and 2-1 (13), is also unknown. It comigrates with L_{BC}-P2 (Table 3), a translation product of L_{BC} which is distinctly different in peptide map from L_{BC}-P1, but which has no identified in vivo equivalent.

VLPs from strain K23, a K1 strain having relatively high L_{BC} levels, can be separated into a lighter ScV-M_1 peak and a heavier ScV-L peak in which ScV-L_{1A} and ScV-L_{BC} are partially resolved. The ScV-M_1 peak contained only VL_{1A}-P1, confirming the role of this capsid in M_1 maintenance (13) and consistent with the inability of VL_{BC}-P1 to perform this function. The VL_{1A}-P1 and VL_{BC}-P1 peaks in the ScV-L peak coincided with the L_{1A}- and L_{BC}-dsRNA peaks, respectively, suggesting that each L-dsRNA is encapsidated only by its homologous capsid protein (El Sherbeini et al., submitted for publication). This has been clearly demonstrated by Thiele et al. (133), who fractionated the VLPs of strain NK3 [KIL-s_1]. Heavy VLPs contained L_{1A}- and S3-dsRNAs (not fractionated) and VL_{1A}-P1. A very light VLP peak contained L_{BC} and was found to contain three smaller proteins, essentially as previously reported for L_{BC} VLPs by Sommer and Wickner (129). It seems likely that the low density of the VLPs containing L_{BC}-dsRNA, which were found near the top of the gradient, was due to damage, for which the protease sensitivity of L_{BC}-P1 may have been responsible.

In a similar experiment, it was shown that the dsRNA transcriptase activity in the light VLP peak from a K1 killer was much more heat labile than in the heavier peak (87). On the assumption that this peak contained ScV-M_1, this result was postulated to explain the heat curability of ScV-M_1. However, these VLPs were almost certainly damaged forms of ScV-L_{BC}.

NUCLEAR MUTATIONS AFFECTING M-dsRNA MAINTENANCE

Maintenance of Killer (*mak*) Genes: the *KRB1* Mutation

By screening K1 killer strains for (K^- R^-) segregants, easily scored by replicating onto a lawn of sensitive cells (Fig. 1), mutants in 30 nuclear loci (*spe2*, *spe10*, *pet18*, *mak1*, *mak3-28*) that cause loss of M_1-dsRNA have been identified (96, 139a, 150, 155, 162, 165). This screen will also include *kex* mutants (see "Structure of the M_1-dsRNA Preprotoxin Gene and Steps in Toxin Maturation"), but unlike *mak* mutants, *kex* mutants retain immunity, also easily scored as in Fig. 1. Many *mak* mutants are marked by a single allele, and it is estimated (156) that 100 such loci may exist, a surprisingly high number. They are not clustered and have been mapped to 15 of the 16 known yeast chromosomes (165).

Because the presence of L_A or L_{BC} confers no obvious phenotype on a sensitive *S. cerevisiae* strain lacking M-dsRNA, their loss cannot be so easily monitored. However, the dependence of M on L_A-dsRNA allows detection, in killers, of mutants required by both M and L_A or whose primary defect is in L_A-dsRNA maintenance. Three of the killer maintenance mutants (*pet18*, *mak3*, and *mak10*) have recently been found to fall into this category (see next section). None of the *MAK* genes is required for L_{BC} or W (see "T-, W-, and XL-dsRNAs").

Although conditional mutants were not deliberately sought, several *mak* mutants result in slow growth (*spe2*, *pet18*, *mak13,15,17,20,22,27*), whereas *mak1*, *mak16*, *mak30*, and *pet18* alleles are temperature sensitive for growth and *mak6* is cold sensitive (156, 165). *MAK6* has recently been identified with a previously known locus, *LTS5*, causing growth to be cold sensitive at 8°C (114). A few conditional (temperature-sensitive) *mak* mutants were found in a deliberate search (50). Thus, M_1-dsRNA maintenance is critically dependent on the function of a number of nuclear

TABLE 7. Summary of biochemical properties and known genetic requirements of *S. cerevisiae* dsRNA types[a]

dsRNA species	[HOK], etc.	Size (kb)	Hybridizes to:	Encodes	*PET18 MAK3,10*	*MAK1,8 SPE2*	*SPE10 MAK27*	*MKT1,2*	CLO$^+$	Temp
L_{1A}	$L_{A\text{-}HN}$	4.7	$L_{1A(++)}$	Capsid	+					ts
L_{2A}	$L_{A\text{-}H}$	4.7	$L_{1A(+)}$	Capsid	+					ts
	$L_{A\text{-}HE}$	4.7	NT	Capsid	+					ts
	$L_{A\text{-}E}$	4.7	NT	Capsid	+		+			ts
L_{BC}		4.7	L_{BC}	Capsid					+	
T		2.7	T	?						TI
W		2.25	W	?						TI
M_1		1.9	M_1	Preprotoxin	+	+	+			ts
M_2		1.7	NT	Preprotoxin	+	+	+	+		ts

[a] Characteristics listed include associated [HOK], [NEX], and [EXL] phenotypes (see text, "[HOK], [EXL], and [NEX]: variations in ability of L_A to maintain M-dsRNAs"), indicated by HN and E suffixes, and positive hybridization interactions. Only those probes listed have been tested, each against all species listed, indicating also the nonhomology between M_1, L_{1A}, L_{BC}, T, and W. Nuclear genes required for maintenance in *SKI*$^+$ hosts are indicated by + (described in text, "Nuclear Mutations affecting M-dsRNA Maintenance"; "Effects of *mak* and *ski* Mutations on L-dsRNA Maintenance"; "T-, W-, and XL-ds-RNAs"). "Temp" indicates temperature sensitivity of maintenance: ts, loss (curing) at high temperature; TI, enhancement at high temperature. NT, Not tested.

genes whose products are important or essential for cell growth.

PET18 is required for M_1-dsRNA maintenance and also for maintenance of mitochondrial DNA (84); other *pet* mutations tested (a large number are now known) do not lead to loss of M-dsRNA.

Genes of known function required for M_1 maintenance include *SPE2*, which encodes adenosylmethionine decarboxylase, required for spermine and spermidine biosynthesis (35), and *SPE10*, which encodes ornithine decarboxylase, required for synthesis of putrescine (139a). Cells grow slowly (but fail to undergo meiosis or to sporulate) in complete absence of *SPE2* function. Both *spe2* and *spe10* mutants lose M_1-dsRNA. No other dsRNAs are affected, except for the loss of $L_{A\text{-}E}$, an unusual L_A variant (see "[HOK], [EXL], and [NEX] . . .," subsection "[EXL], $L_{A\text{-}E}$, and $L_{A\text{-}HE}$"), in *spe10* mutants (139a). This variant, unlike all other L-dsRNAs, also requires *MAK27* (159). The physical characteristics of the different *S. cerevisiae* dsRNAs and their differential chromosomal gene requirements are listed in Table 7

MAK8 is identical to *TCM1* (166), the gene for trichodermin resistance, encoding ribosomal protein L3. Some function of this protein is apparently more critical for M_1-dsRNA maintenance than for growth. This function could involve translation, as part of the ribosome, or might, for example, involve direct interaction of protein L3 with M_1-dsRNA or its transcripts, just as the *E. coli* translational factors Tu, Ts, and S1 are involved in replication of Qβ RNA phage (2).

MAK1 has recently been identified with the gene for yeast topoisomerase-1 (136). Screening of extracts of temperature-sensitive mutants identified a mutant with a topoisomerase-1 (temperature-sensitive) defect. This was found to be allelic with *mak1-1*, which has less than 1% wild-type topoisomerase-1 activity by this in vitro assay, a surprising result.

Loss of functional mitochondrial DNA bypasses the requirement for *MAK10* for maintenance of M_1-dsRNA; antibiotic-induced respiratory deficiency does not (154). A dominant nuclear mutation in the *KRB1* locus, isolated as a suppressor of *mak7*, also bypasses *pet18* (164) (Table 8), but not several other *mak* mutations. Although loss of M_1-dsRNA is prevented in *pet18 KRB1* strains, mitochondrial DNA loss is not prevented. The interplay between these nuclear loci, mitochondrial DNA, and M_1-dsRNA maintenance is not understood. *KRB1* is centromere linked, but is not located on any of the 16 known yeast chromosomes. It thus defines a new chromosome, XVII (161).

Superkiller (*ski*) Mutants and the [KIL-b] Plasmid Mutant

The same plate test for killer zone size (Fig. 1) which allows detection of (K^- R^-) *mak* mutants derived from (K^+ R^+) killers also allows identification of "superkiller" (K^{++} R^+) strains in which the zone of killing is larger than normal. Such strains fall into several categories, as follows:

(i) loss of toxin cell wall receptor (*kre1,2* mutants; see "Mode of Action of Killer Toxin") resulting in escape of a larger fraction of toxin produced into the medium (32);

(ii) a *ski5* mutation resulting in loss of a toxin-degrading exocellular protease and increased toxin accumulation (see "Mode of Action of Killer Toxin"; 31);

(iii) a [KIL-sk_1] mutation in M_1-dsRNA, as in strain T158c, resulting in production of a more stable toxin, presumably less sensitive to the *ski5* gene product;

(iv) a [KIL-b_1] plasmid mutation, presumed but not proven to reside in M_1-dsRNA, which results in an increased M_1-dsRNA copy number (139; R. B. Wickner, personal communication) and is presumably responsible for increased toxin production (common in laboratory yeast strains);

(v) mutations in nuclear *ski2,3,4,6,7,8* loci which also increase the M_1-dsRNA copy number (and also that of L-dsRNAs; see next section) (114, 139); and

(vi) a *ski1* mutation, which increases toxin production without an effect on M_1-dsRNA copy number. Unlike other *ski* mutations, *ski1* fails to suppress *mkt* ("[HOK], [EXL], and [NEX] . . .," subsection "[NEX], $L_{A\text{-}HN}$, and *mkt1,2*").

The *ski1,2,3,4* mutants were first identified by this superkiller phenotype and later found to suppress certain *mak* mutations (139). The *ski5,6,7,8* mutants were later discovered by a second phenotype, suppression of *mkt* [see section cited in (iv) above]. Their effects on *mak* mutations have not yet been described. The [KIL-b_1] plasmid mutation also "bypasses" a requirement for certain intact *MAK* genes

TABLE 8. Classification of *MAK* and *ski* mutations[a]

mak group	Genes	Suppressed by:			
		ski1	*ski2,3,4*	[*KIL-b*]	*KRB1*
MI	*mak16*				
MII	*mak3,10 pet18*	+			
MIII	*mak12,21,26*	+	+		
MIV	*mak1,4,6,7,11 spe2*	+	+	+	
	mak7 pet18	+	+	+	+

[a] *ski6,7,8* (see text) have not yet been fitted into this scheme.

(139); the classification of this plasmid mutation, *KRB1*, and of *mak1-4* mutations with respect to suppression of *mak* phenotype is shown in Table 8. None suppress *mak16*; the three genes required for L_{1A} maintenance are suppressed only by *ski1*; *ski2,3,4* suppress all of those suppressed by [KIL-b_1], plus *mak12,21,26* (160). All *ski* mutants (except *ski5*), suppress the other *mak* mutations.

In certain killers, a cytoplasmically inheritable phenotype is found in which M_1-dsRNA maintenance is dependent on a *ski* mutation. This was originally attributed to a variant in the M_1-dsRNA itself and given the name [KIL-sd_1] (138). It is now known to be due to the presence of an L-dsRNA variant, $L_{A\text{-}E}$, in these strains. $L_{A\text{-}E}$ is unable to maintain M_1-dsRNA in a *SKI*$^+$ background, but is competent in *ski*$^-$ (see "[HOK], [EXL], and [NEX]: Variations in Ability of L_A to Maintain M-dsRNAs").

Since *mak* mutations include the topoisomerase, *pet18*, *tcm1*, and *spe2* mutations, in genes of widely different function, speculation on common functional interactions between the products of *MAK* and *SKI* genes seems to be premature. However, the growth-retarding or conditional nature of many of these mutations and the complex pattern of nuclear-dsRNA plasmid interactions implied by this genetic analysis do indicate the profundity of the integration of maintenance of M_1-dsRNA, and to a lesser extent of L_A-dsRNA, with essential cellular maintenance machineries. Some of this complexity might derive from exploitation by the dsRNAs of cell regulatory systems involving direct interaction of multiple regulatory gene products, in the type of regulatory protein complex postulated to govern expression of the yeast acid phosphatase genes (137). A likely focus of this regulation is initiation of dsRNA replication by second-strand synthesis on a primary plus-strand transcript (Fig. 3). Interactions with different dsRNAs are discussed in "T-, W-, and XL-dsRNAs."

Significance of *mak* and *ski* (etc.) Mutations

The mutations listed in Table 7 are of direct value in studies of the killer system since they can be used to manipulate cellular dsRNA contents. Mutations affecting toxin secretion, such as *kre1* and *-2*, *ski5*, and *kex1* and *-2* (see "Structure of the M_1-dsRNA Preprotoxin Gene and Steps in Toxin Maturation"), are of considerable interest to individuals attempting to analyze and optimize protein secretion in *S. cerevisiae*. It seems likely that the *SKI* loci (other than *ski5*) are involved in relatively general, negative regulation of dsRNA replication (see "[HOK], [EXL], and [NEX] . . .," subsection "Conclusion"). The value of other mutations may derive principally from analysis of their effects on basic cell metabolism, as illustrated by the *spe*, *tcm*, and topoisomerase 1 mutations.

It appears that identification of loci affecting killer phenotype allows isolation of mutations apparently partially defective in a wide variety of fundamentally important nuclear gene products. This is valuable only after identification of the primary defect, a haphazard process so far, involving the demonstration of allelism between mutants isolated for apparently independent phenotypes. However, it seems possible that any *mak* or other killer mutation resulting in retarded or temperature-sensitive growth may be worthy of biochemical analysis if clues to the primary defect can be elucidated.

Wickner (156) has pointed out that retarded growth is not, by itself, responsible for M_1-dsRNA loss in *mak* mutants. For example, a *spe2 ski2* mutant still grows slowly, but retains M_1. Similarly, a *pet18 ski1* strain remains petite and temperature sensitive for growth although competent for M_1 maintenance.

EFFECT OF *mak* AND *ski* MUTATIONS ON L-dsRNA MAINTENANCE

Until recently, curing of M_1-dsRNA or loss due to introduction of a *mak* mutation was not thought to cause loss of L-dsRNA, although a reduction by about threefold was observed on introduction of *mak3* into a K1 killer (165). One killer strain, K7, was known to be an exception since introduction of *mak10* led to loss of both M_1- and L-dsRNAs (96). It is now known that this reflects the dependence of L_A-dsRNA maintenance on *MAK3*, *MAK10*, and *PET18* (41, 129) and that this is the only L species in strain K7; the residual L-dsRNA in other strains is L_{BC}, which is independent of these genes (Table 7). The loss of all L-dsRNA from strain K7 and the loss of a major fraction of the total L-dsRNA in other K1 killers during heat curing of the killer phenotype (151) is similarly due to the temperature sensitivity of L_A but not of L_{BC} maintenance (Table 7). M_1 is heat cured more readily than L_A (128) and may have a more stringent requirement than L_A for an unidentified temperature-labile cell component, or it may simply require a high L_A copy number for its maintenance.

In these experiments (128), it was shown that, after heat curing of a K1 killer to the point that L_A was eliminated from 90% and M_1 from 99% of the progeny, the few remaining killer clones (retaining M_1) all retained L_A. This is the most stringent demonstration of the dependence of M_1 on L_A.

Maintenance of M_2-dsRNA in strains containing L_{2A} is known to require *MAK10* (97). By analogy with the K1 system, this suggests that L_{2A} shares with L_{1A} at least the requirement for *MAK10*. M_2 also requires *MAK8* and *MAK*16. Other *mak* loci have not been tested (160). M_2 is heat curable and resembles M_1 in all characteristics tested, except in its requirement for *MKT* (see next section, subsection "[NEX], $L_{A\text{-}HN}$, and *mkt1,2*"). No data on M_3 have been reported. A variant of L_A, $L_{A\text{-}E}$ (see next section), is atypical in being also dependent on *MAK27* (159).

[HOK], [EXL], AND [NEX]: VARIATIONS IN ABILITY OF L_A TO MAINTAIN M_1-dsRNAs

Several variants of L_A-dsRNA have been identified by Wickner and co-workers according to their ability to maintain or exclude M_2- and M_1-dsRNAs in different genetic backgrounds (128, 129, 157, 159, 167). These are listed in Table 9 together with their associated plasmid phenotypes, [HOK], [NEX], and [EXL], and some of their critical properties. These cytoplasmically inherited phenotypes were initially defined as unidentified plasmids but are now known to be phenotypic variants of the L_A-dsRNA plasmid. L_{1A}, the dsRNA normally found in K1 killers, carries [HOK] and [NEX] phenotypes and so is called $L_{A\text{-}HN}$. L_{2A}, on the other hand, has only weak [HOK] activity (159) and lacks [NEX] function. It can mutate to $L_{2A\text{-}H}$, with strong [HOK] and (EXL^R) activity (159) (see below). $L_{A\text{-}E}$ and $L_{A\text{-}HE}$ are L-dsRNA species with unique properties which were found in particular sensitive strains (AN33 and 200, respectively; 157, 159) with no known descent from K1 or K2 killers. These forms of L_A seem to be part of a continuous spectrum in their ability to maintain M_1- and M_2-dsRNAs (Table 9). All seem less competent for M_2- than for M_1-dsRNA maintenance.

TABLE 9. Competence of L_A-dsRNA variants for M-dsRNA maintenance[a]

L_A variant[b]	Genotype	M_1-dsRNA			M_2-dsRNA (*MKT*$^+$ strains)		
		SKI$^+$ ($L_{A\text{-}E}$)	*SKI*$^+$	*ski*$^-$	*SKI*$^+$ ($L_{A\text{-}E}$)	*SKI*$^+$	*ski*$^-$
$L_{A\text{-}E}$	[EXL]		−	± (low)		−	ND
$L_{A\text{-}HE}$	[HOK] [EXL]		+	+		−	+
L_{2A}	[HOK^w]	±	+	++	−	+	+
$L_{A\text{-}H}$	[HOK]	+	+	+++	−	+	++
$L_{2A\text{-}H}$	[EXL^r] [HOK]	ND	ND	ND	+	+	++
$L_{A\text{-}HN}$	[HOK] [NEX]	+	+	+++	+	+	++

[a] The copy number of M-dsRNA maintained ranges from zero (−) through low (±), moderate (+), to high (++). ND, Not determined.

[b] $L_{A\text{-}E}$ = Incompetent for maintenance of M_1- or M_2-dsRNA in a *SKI*$^+$ host; excludes M_2-dsRNA from combination with L_{2A} and maintains M_1-dsRNA in a *ski*$^-$ host at low copy number, a cold-resistant combination; $L_{A\text{-}HE}$ = Competent for maintenance of M_1- but not M_2-dsRNA in a *SKI*$^+$ host; excludes M_2-dsRNA from combination with L_{2A}. L_{2A} = Competent for maintenance of M_1 and M_2-dsRNA in a *SKI*$^+$ host at low copy number, but maintenance of M_2 is recessive to $L_{A\text{-}E}$; weak ([HOK^w]) $L_{2A\text{-}H}$ = (EXL^R) mutant of L_{2A}; now dominant to $L_{A\text{-}E}$ for maintenance of both M_2 and M_1-dsRNAs; strong [HOK]. $L_{A\text{-}HN}$ (L_{1A}) = Competent for maintenance of M_1- and M_2-dsRNAs at moderate copy numbers in *SKI*$^+$ hosts (the high levels of M_1-dsRNA supported in *ski*$^-$ hosts leads to cold sensitivity); prevents exclusion of M_2 by $L_{A\text{-}E}$, but excludes M_2 in a *SKI*$^+$ *mkt*$^-$ host.

[HOK] and $L_{A\text{-}H}$

The form of L_A-dsRNA that is competent for the maintenance of M_1- or M_2-dsRNA in a *SKI*$^+$ nuclear background (Table 9) is called $L_{A\text{-}H}$, where the H subscript stands for [HOK], a cytoplasmically inherited genotype originally called Help Of Killer (157), because of its ability to maintain a purported M_1-dsRNA mutant called [KIL-sd$_1$] for *ski* dependent (167) (see below).

[EXL], $L_{A\text{-}E}$, and $L_{A\text{-}HE}$

A sensitive yeast strain, AN33, was found to harbor a form of L-dsRNA that, on mating with a K2 killer (carrying M_2- and L_{2A}-dsRNAs) resulted in exclusion of the M_2-dsRNA, producing a sensitive diploid (157). The AN33 plasmid was called [EXL] and later found to be a form of L_A-dsRNA now called $L_{A\text{-}E}$ (129, 157, 159).

It is now known that $L_{A\text{-}E}$ is incompetent for the maintenance of either M_1- or M_2-dsRNA in a *SKI*$^+$ background (Table 9). In a *ski*$^-$ background, it is able to maintain M_1-dsRNA. [KIL-sd$_1$] is now known to be a combination of normal M_1 with $L_{A\text{-}E}$-dsRNA and a *ski*$^-$ mutation. Thus, the test for [HOK] is introduction into a *SKI*$^+$ strain of $L_{A\text{-}E}$ and M_1 from a *ski* host. Maintenance of the K_1^+ R_1^+ phenotype in the diploid indicates the presence of $L_{A\text{-}H}$ in the *SKI*$^+$ strain, since *SKI*$^+$ is dominant. Since introduction of $L_{A\text{-}H}$ renders the M_1-dsRNA in such strains independent of the *ski* mutation, $L_{A\text{-}H}$ is either replicated with or excludes $L_{A\text{-}E}$. Conversely, the ability of $L_{A\text{-}E}$ to exclude M_2 on mating to a K2 killer means that $L_{A\text{-}E}$ excludes L_{2A} or is dominant in its effect on M_2-dsRNA maintenance. It is now known (159; Ball and Wickner, in press; Wickner, personal communication) that L_{2A} has weak [HOK] activity, being able to maintain both itself and either M_2- or M_1-dsRNA at relatively low copy numbers in a *SKI*$^+$ host. A *SKI*$^+$ diploid produced by mating of an $L_{A\text{-}E}$ strain with a strain in which M_1 was maintained by L_{2A} is a stable, weak killer retaining low M_1 content (Wickner, personal communication). Presumably, it also retained low L_{2A} content. Possibly a reduction in L_{2A} on introduction of $L_{A\text{-}E}$ is sufficient to cause complete loss of M_2 but only reduction in M_1-dsRNA.

$L_{A\text{-}HE}$ is the L-dsRNA found in strain 200 (159). It excludes M_2 on mating with a K2 killer ([EXL] phenotype), but is capable of maintaining M_1-dsRNA at moderate copy number in a *SKI*$^+$ background ([HOK] phenotype). It will maintain both in a *ski*$^-$ background.

L_{2A} and $L_{2A\text{-}H}$ (EXL^R)

The L_A found in natural K2 killers has weak [HOK] activity. Introduction of $L_{A\text{-}E}$ causes loss of M_2. After mutagenesis of such a K2 strain, (EXL^R) variants resistant to M_2 exclusion by $L_{A\text{-}E}$ were obtained (159). They were found to have acquired a strong [HOK] phenotype in their L-dsRNA, which has tentatively been called $L_{2A\text{-}H}$ (Table 9).

[NEX], $L_{A\text{-}HN}$, and *mkt1,2*

L_{1A}, the L_A-dsRNA normally found in K1 killers, has strong [HOK] activity. Also, when associated with M_2-dsRNA, it prevents exclusion by [EXL] ($L_{A\text{-}E}$). This phenotype is called [NEX] and this L_A type is called $L_{A\text{-}HN}$ (163). L_{1A} is, therefore, $L_{1A\text{-}HN}$. $L_{A\text{-}HN}$ is also the L_A species found in sensitive strains S288c and S7, which have been much used in killer studies. It is the only L-dsRNA found in strain S7 and killer strains K7, K12, and K23.

$L_{A\text{-}HN}$ itself excludes M_2 in crosses with K2 killers containing L_{2A} if the haploid segregant contains a defect in either of two nuclear loci called *mkt1* and *mkt2*. Exclusion occurs at 30°C, but not at 20°C (157, 159). The $L_{2A\text{-}H}$ mutant form of L_{2A} has the (EXL^R) part of the [NEX] phenotype, but fails to cause exclusion of M_2 in an *mkt*$^-$ strain.

Exclusion of M_2 by $L_{A\text{-}HN}$ in an *mkt*$^-$ strain is prevented by a *ski2*, *-3*, or *-4* mutation, but not by *ski1*. Selection for further suppressors of M_2 exclusion has led to the discovery of three further *ski*$^-$ loci called *ski6*, *-7*, and *-8* (114). Mutations in any of these six *ski* loci lead to a marked increase in L_A, L_{BC}, and M_1-dsRNA copy number (Ball et al., in press). An $L_{A\text{-}HN}$ M_1 *ski*$^-$ combination causes cold sensitivity which is lost if M_1 is removed. This suggests that M_1 replication requires some cell component critical for growth at low temperature, such as the *LTS5* gene product, that is titrated by a high M_1 copy number (114). If $L_{A\text{-}E}$ replaces $L_{A\text{-}HN}$ in a *ski*$^-$ strain, M_1 copy number is low and the strain is cold resistant. It has also been observed that the presence of M_1-dsRNA causes a marked reduction in L_A copy number (Ball et al., in press).

Mutations in three further loci, called *mks1*, *mks2*, and *MKS50*, were found to suppress [NEX] exclusion of M_2 in *mkt* strains. These mutants, however, did not portray other aspects of the *ski* phenotype (114).

Exclusion of M_2- by M_1-dsRNA

Besides exclusion of M_2-dsRNA by [EXL], or by [NEX] in an *mkt*$^-$ background, M_2- is excluded by M_1-dsRNA (98). This is unrelated to the $L_{1A\text{-}HN}$-dsRNA also present in K_1 killers, since cells cured of M_1-dsRNA, but retaining $L_{1A\text{-}HN}$-dsRNA, no longer exclude M_2 (159).

Conclusion: Mechanisms of Exclusion and Models for Control of dsRNA replication

At present, no coherent explanation exists for the interactions of L-dsRNAs with themselves, with M_1- and M_2-dsRNAs, and with the products of *SKI*, *MAK*, and *MKT* loci. The differential effects of mutations in these loci on maintenance of different dsRNAs (Table 7) indicate significant differences in mechanisms of replication or its control.

At the same time, the increase in copy number of M_1, M_2, L_A-, and L_{BC}-dsRNAs caused by *ski* mutations suggests convergence on a pathway negatively controlled by *SKI* gene products (Ball et al., in press). Data (our unpublished observations) suggesting mutual down-regulation of L_A and L_{BC} similarly suggest competition in replication. It would be surprising if the dsRNAs failed to share common chromosomally encoded replication components. Nevertheless, a chromosomal defect (clo^-) in L_{BC} replication has only recently been recognized (149), and no mutant causing loss of W- or T-dsRNAs is known (see next section). The known maintenance mutants can be organized into a converging hierarchy (Fig. 4), as suggested by Wickner (160). The bias towards recognition of mutations affecting M- or L_A-dsRNAs clearly reflects the ease with which their loss can be detected. One suspects that most of the genetic loci indicated only modulate the efficiency of the actual replication event and that this scheme should not be regarded as a biosynthetic pathway. It is not known whether *MAK3,10* and *PET18* are required directly only by L_A, or also by M_1.

The dependence of M_1 on L_A (Fig. 4, positive arrow) seems clearly related to the provision of capsid peptide. Ball et al. (in press) suggest, alternatively, that L_A helps M_1 maintenance by antagonizing the action of *SKI* gene products. The reduction of $L_{A\text{-}HN}$ in the presence of high M_1 in a ski^- strain (114) (Fig. 4, negative arrow) may reflect competition for a limited capsid supply but might equally reflect competition for an unidentified limiting cellular component, such as the *MAK 3, 10* or *PET18* gene product. Investigation of the effects of mixing various combinations of $L_{A\text{-}E}$, L_{2A}, $L_{A\text{-}HN}$, and L_{BC} on their respective copy numbers, in identical nuclear genetic backgrounds, would clarify some of these issues. The permutations of presence of M-dsRNAs, *ski*, *mak*, and *mkt* mutations are obviously large. However, specific issues should soon be resolved. For example, is L_{2A} excluded by $L_{A\text{-}E}$ and is $L_{A\text{-}E}$ itself excluded by $L_{A\text{-}HN}$?

If recognition by capsid is involved in initiation or elongation events in dsRNA synthesis (14), then exclusion phenomena could arise from competition for capsid between different transcripts, or from abortive binding of nonhomologous capsid. This implies that [HOK], [NEX], and [EXL] phenotypes are aspects of capsid structure. This may become clear upon comparison of capsids and capsid genes in VLPs containing $L_{A\text{-}E}$, $L_{A\text{-}HN}$, and other L_A species. The quite distinct peptide maps for L_{1A}-P1 and L_{2A}-P1, however (see above, "Role of L-dsRNA Species in Capsid Production and M_1-dsRNA Maintenance"), suggest that results may be difficult to interpret, since both have, or can apparently acquire, M_1-specific replication functions implied by the [HOK] genotype. It seems equally likely that exclusion phenomena are due to selective competition between dsRNA species for nuclear gene products required for dsRNA maintenance. For example, $L_{A\text{-}HN}$ and M_2 may both require *MKT* gene products, although these are not required (directly) by M_1. Similarly, M_1 and M_2 may require capsid or an unidentified gene product, for which M_1 competes most effectively. This type of competition may also explain the phenotype of suppressive-deletion mutants of M_1-dsRNAs ("Summary of Phenotypes . . .," subsection "Plasmid Mutations in M_1-dsRNA").

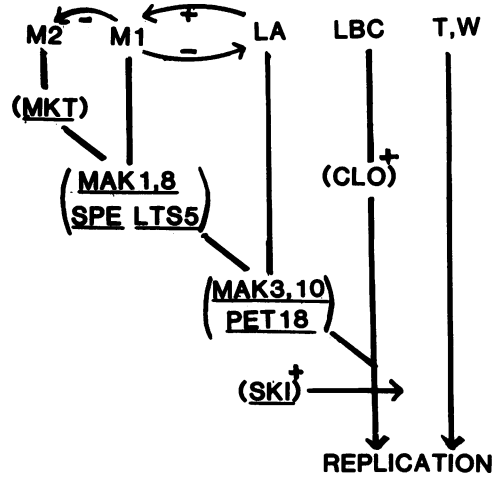

FIG. 4. Schematic replication pathway illustrating the genetic requirements of different dsRNAs. All genes are involved in positive regulation except for the SKI^+ genes. The positive role of L_A on M_1 replication is, at least in part, provided by capsid. The negative effect of M_1 on L_A copy number is marked in *ski2* mutants (Ball et al., in press). No mutations affecting T or W replication are known.

The variation in properties of L_A found in different strains may reflect either discrete types or the products of rapid divergent evolution of the dsRNA genome (see "Evolution of the Killer System"). Thus, $L_{A\text{-}E}$ may have evolved for self-maintenance in the absence of a requirement for M_1 maintenance, whereas L_{1A} ($L_{A\text{-}HN}$) has been selected for M_1 maintenance and L_{2A} has been selected for M_2 maintenance. The production of quantities of K2 toxin sufficient for competition with other yeast strains may require only a relatively low M_2 copy number.

T-, W-, and XL-dsRNAs clo$^-$ and the Discovery of T and W

Discovery of a complex nuclear chromosomal defect called clo^-, which results in failure to maintain L_{BC} at 25°C (maintenance persists at 30°C or higher temperatures), has allowed study of two low-copy-number dsRNA species, unrelated to L and M-dsRNAs, called T and W (149).

Vodkin (141) unexpectedly obtained strains lacking all detectable dsRNA (including L, M, T, and W) by heavy ethyl methanesulfonate mutagenesis of killer strain FM11 [PSI^+], selection for [PSI^+] phenotype (cytoplasmically inherited enhancement of nonsense suppression), and screening for nonkillers. One such strain, JM6 (141), has recently been found (149) to give a second unexpected result: 2 to 2 segregation of progeny retaining or losing L-dsRNA when crossed with a *pet18* strain presumably containing L_{BC}. Strain 1815, a segregant of this cross lacking L-dsRNA (L-o phenotype), contains the clo^- ("chromosomal L-o") defect which results in frequent segregation of L-o meiotic progeny in crosses to certain strains containing L_B but not in crosses to strains carrying L_A or L_C. Because clo^- depends on at least two unlinked chromosomal defects, a *kar1* clo^- L-o strain was constructed and used to test the dependence of various dsRNAs on CLO^+. L_B, L_C, and L_{BC} but not L_A, M_1, or M_2 were found to require CLO^+ by this test. The lack of consistency with the meiotic data on L_C is not understood (149).

Electrophoretic analysis of the dsRNAs isolated from clo^- L-o strains (149) showed the persistence or enhancement of two unique bands of 2.7 and 2.25 kb, migrating between L- and M-dsRNAs, whose presence has frequently been noted but whose independence of M and L was not previously apparent. They have been called T (2.7 kb) and W (2.25 kb). They are inherited cytoplasmically and strains have been found carrying neither, both, or only W. Thus, T may be

dependent on W, but neither is dependent on any L-dsRNA. Maintenance of W is independent of 25 *MAK* genes tested, and neither T nor W shows cross-hybridization to each other or to any of the known L- and M-dsRNAs. The copy numbers of both are increased about 10-fold by growth at 37°C, except in strains carrying an unidentified cytoplasmic gene. Encapsidation has not been demonstrated; however, a sucrose gradient peak of T-dsRNA was observed in a crude extract of cells of a strain containing both T and W, migrating between ScV-L and free T-dsRNA (149).

Independent dsRNA Families and Their Roles in *S. cerevisiae*

At least three apparently independent dsRNA families are now recognized in *S. cerevisiae*: L_A-dsRNAs and their M-dsRNA satellites, L_{BC}-dsRNAs, and T and W. Other dsRNA species may exist, and minor bands in addition to T and W are frequently seen on electrophoresis of dsRNA preparations, migrating between L and M. XL, another minor species, is sometimes observed migrating slightly slower than L. The relationship of these species to other dsRNAs is unknown.

None of the three dsRNA families correlates with mitochondrial DNA, the 2μm plasmid, [URE3], [PSI], 20S RNA, or other cytoplasmically inherited phenotypes or genomes (149, 160), and none of them is apparently required for cell maintenance in the laboratory. Their role in the wild is also unknown and their prevalence has not been studied. It is possible that wild-type homothallic yeasts may segregate strains containing unique dsRNA species which have only become mixed in the laboratory. The evidence on "wild-type" *Saccharomyces* sp. strains is fragmentary, but suggests no pattern. Thus, most wild-type strains seem to contain L_{BC}, W, and frequently T and L_A. Exceptions are S3, a *mak10* L-o strain (T and W content unknown; 127), and the *S. carlsbergensis* strains Y3795D and NCYC CB11 which lack 2-μm circular DNA and detectable dsRNA. If these are competent in the wild, then the various dsRNA species may be merely parasitic, tolerated entities lacking survival value. The origin of the satellite killer M-dsRNAs is a separate mystery (see "Evolution of the Killer System").

STRUCTURE OF THE M_1-dsRNA PREPROTOXIN GENE AND STEPS IN TOXIN MATURATION

Toxin Is Derived from a Glycosylated Intracellular Protoxin

The only radiolabeled species specifically immunoprecipitated from extracts of pulse-labeled cells of K1 killers by antitoxin immunoglobulin G is a 42- to 44-kd species (12). This species is chased into exocellular toxin with a half-life of about 25 min at 30°C (24, 25) and so is a protoxin. This protoxin is converted to a 34- to 35-kd species by endoglycosidase H (12). Since this enzyme cleaves the di-*N*-acetyl chitobiose units that link the oligomannosyl side chains in eucaryotic glycoproteins to asparaginyl residues (132), protoxin is a glycoprotein of this type. This is confirmed by the effects of tunicamycin, an inhibitor of the synthesis of the glycosyl donor for this type of protein modification, on protoxin synthesis. Preincubation of cells with tunicamycin (to exhaust donor) and pulse-labeling reveal a modified protoxin of about 34 to 35 kd. This is unstable, but chases only poorly into exocellular toxin (25). Inhibition of glycosylation apparently prevents efficient toxin secretion and labilizes the protoxin to intracellular degradation.

Neither α nor β toxin component is glycosylated (107), so that the glycosylated portion of protoxin must be removed during maturation. A model of toxin maturation (see below) is shown in Fig. 5.

Toxin Is Encoded by M_1-dsRNA Which Also Determines Immunity

Genetic evidence clearly demonstrates M_1-dsRNA to be the determinant of both toxin production and immunity. First, both phenotypes are lost concomitant with loss of M_1-dsRNA by curing (heat, cycloheximide, etc.) (46, 95, 151). Second, induced plasmid mutants include both neutral and suicidal types (24). Like naturally isolated killers and neutral mutants, these phenotypes correlate with M_1-dsRNA presence in curing and transmission experiments (7, 24).

The identity of M_1-dsRNA and the killer determinant was confirmed by demonstrating that M_1-P1, the 34-kd single polypeptide comprising more than 90% of the proteins produced from denatured M_1-dsRNA by a rabbit reticulocyte translation system, contains all of the 20 to 23 *Staphylococcus aureus* V8 protease-generated peptide components of secreted toxin (11). The cDNA sequence predicts 24 V8 peptides in mature toxin (see below). M_1-P1 is very similar in size to deglycosylated protoxin and is specifically immunoprecipitated by antisera raised against purified toxin (13). M_1-P1 is also the product of in vitro translation of both the m and m_a single-stranded in vivo transcripts of M_1-dsRNA (9).

M_1-P1 was predicted to be identical to the primary in vivo translation product of M_1-dsRNA, preprotoxin (11). Since M_1-P1 (34 kd) exceeded the size of toxin (19 kd) and its in vitro assayed leader peptide (estimated at 1.6 kd; see below) by about 13 kd, and since toxin was known to lack glycosylation, it was postulated (11, 12) that a glycosylated fragment of protoxin is the immunity determinant and that M_1-dsRNA is monocistronic.

Protoxin Maturation Involves the Normal Protein Secretion (*sec*) Pathway

The normal pathway for protein secretion in yeasts resembles that in higher eucaryotic cells and is defined by the series of temperature-sensitive *sec* mutants isolated by Novick et al. (102–104). A group including *sec18* accumulates secreted proteins in the endoplasmic reticulum (ER) at 37°C and is blocked in transfer to the Golgi. A single mutant, *sec7*, accumulates protein in Golgi structures and has a leaky block in transfer to secretion vesicles. A third group, including *sec1*, is blocked in post-Golgi transfer from secretion vesicles to the exterior (Fig. 5) (102). Use of these mutants to study the maturation of invertase demonstrates that core glycosylation occurs in the ER and elongation occurs in the Golgi (42). K1 killer strains of *sec18, sec7*, and *sec1* mutants all produce protoxin of apparently normal size at 37°C (25), indicating that most protoxin modifications occur during cotranslational entry into the ER, with none of the massive elongation of the carbohydrate chains in the Golgi common to secreted proteins such as invertase and acid phosphatase (42), even though protoxin accumulation in *sec7* at 37°C indicates Golgi involvement in toxin secretion (Table 10).

The effects of the presence of dog pancreas membrane vesicles, a model of ER function (121), on translation of denatured M_1-dsRNA or of the m and m_a in vivo transcripts are consistent with these in vivo results. M_1-P1 (34 kd) is converted to a mixture of M_1-P1a (about 1.6 kd smaller) and M_1-P1b, the size of protoxin (43 kd) (12). Both species become protected against added protease (25), so are presumably located inside the vesicles. Purified M_1-P1b is converted to M_1-P1a by endoglycosidase H (12). It is probable, therefore, that transport into these vesicles is accompa-

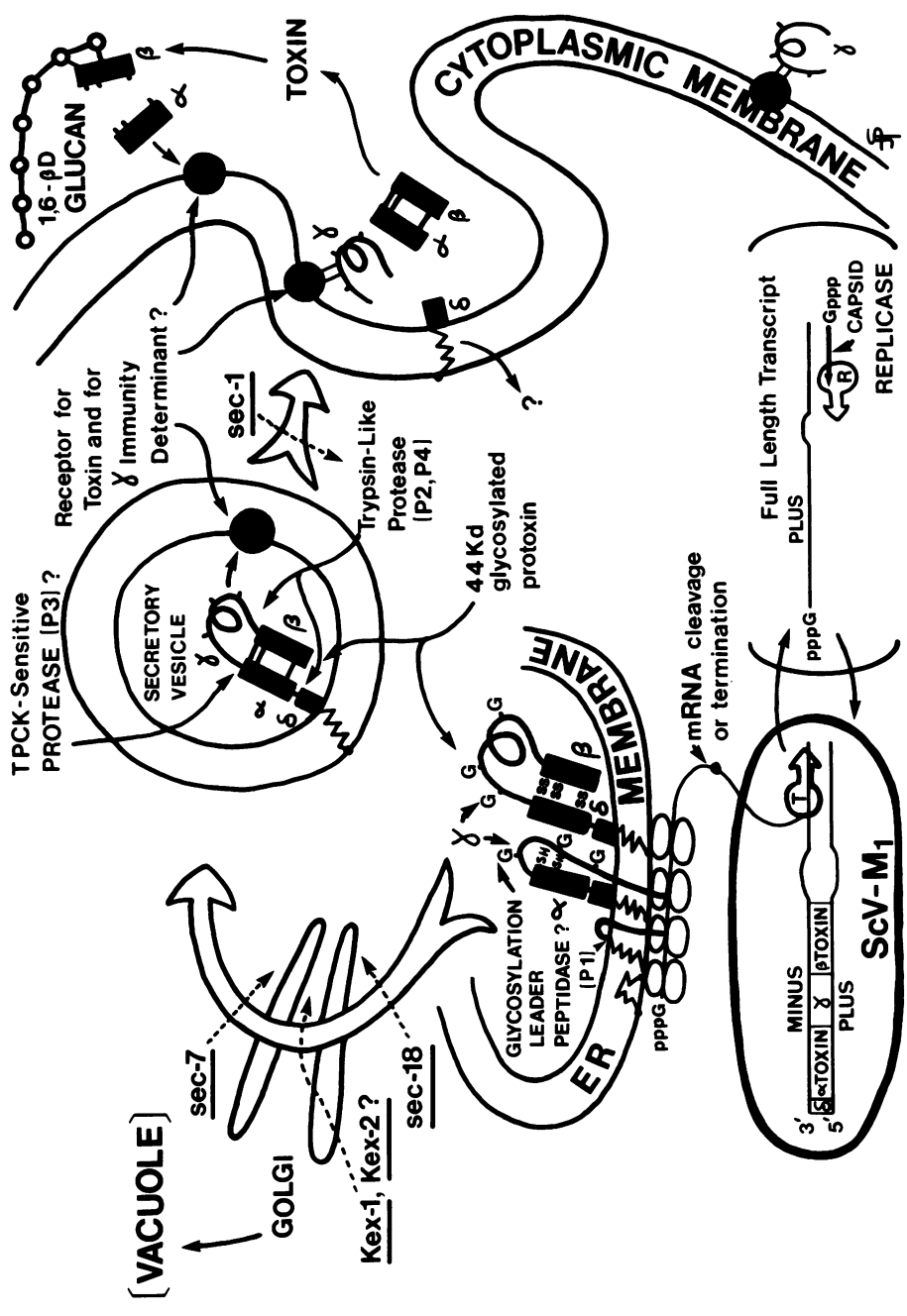

FIG. 5. Proposed maturation of preprotoxin via the yeast secretion pathway. A partial-length plus-strand transcript of M_1-dsRNA is extruded from ScV-M_1 VLPs, associates with ribosomes very near its N terminus, and associates with the ER secretory mechanism by virtue of the hydrophobic leader sequence in the N-terminal δ component of preprotoxin. This component may subsequently anchor the preprotoxin to the membranes of the secretory pathway. Glycosylation of the γ segment occurs in the ER. No known further modifications occur during passage through the Golgi apparatus (defined by the effects of *sec18* and *sec7* mutations), although *KEX1* and *KEX2* gene products may function at this stage. Fragmentation of preprotoxin, involving a TPCK-inhibitable protease, occurs in secretory vesicles, before release of the disulfide-bonded α- and β-toxin components by fusion with the cytoplasmic membrane. The γ component is hypothesized to interact with an unidentified cytoplasmic membrane toxin receptor, rendering the cell immune. The fate of δ is unknown. Toxin attacks sensitive cells by interacting with a 1,6β-D-glucan cell wall receptor, possibly via its β component, labilizing linkage to the α component which interacts with the membrane receptor to create the lethal cation-permeable pores. Full-length transcripts are involved in replication, involving encapsidation of nascent dsRNA as it is formed.

TABLE 10. Nuclear genes affecting expression of K1 toxin and effects of TPCK

Gene	Conditional	K1 killer phenotype	Protoxin half-life (min)[a]		Comments
			−TPCK (temp, °C)	+TPCK	
Wild type		$K_1^+ R_1^+$	25 (30)	~100	Toxin and other proteins secreted normally
kex1	No	$K^- R_1^+$	80 (30)	~100	No toxin secreted; other secreted proteins appear normal
kex2	No	$K^- R_1^+$	15 (30)	15	No toxin or mature α factor secreted; other secreted proteins larger than normal
kex1 kex2	No	$K^- R_1^+$	~100 (30)		Phenotype like *kex1*
sec18	ts (37°C)[b]		80 (37)	~100	Blocks transfer from ER to Golgi
sec7	ts (37°C)		40 (37)	~100	Leaky block in transfer from Golgi to secretory vesicles
sec1	ts (37°C)		15 (37)	~100	Accumulation in secretory vesicles

[a] Data on half-lives are approximate (25).
[b] ts, Temperature sensitive.

nied by removal of a 1.6-kd N-terminal secretion leader or signal peptide by leader peptidase and by asparagine *N*-glycosylation. Consistent with this model (see Fig. 5), both protoxin and mRNA for M_1-P1 were found to be membrane associated in vivo (9).

A more accurate estimate of the size of the peptide removed by these vesicles is derived from translation of S3-dsRNA. The deletion in M_1-P1 which produces S3 initiates after 230 bases, within the α toxin peptide region of the M_1-P1 gene (Fig. 3; see below). The in vitro product of translation of S3, S3-P1, is an 8-kd species that is precipitated by antitoxin immunoglobulin G (12). It is predicted to consist of the first 72 amino acids of M_1-P1, including 28 amino acids of α, and a short C-terminal region derived from sequence at the other side of the deletion, toward the 3′ end of the M_1-dsRNA plus strand (Fig. 3). In the presence of membrane vesicles, S3-P1 is converted to S3-P1a (6.4 kd), which does not become glycosylated. It has the normal initiation and leader sequence but lacks the normal glycosylation sites of M_1-P1. It is undetectable in vivo (12).

These results demonstrate that toxin maturation involves the normal pathway for protein secretion. Secreted yeast glycoproteins such as invertase gain a core $(GlcNAc)_2$-$(Man)_9$-$(Glu)_3$ glycosyl subunit in the ER. The estimated size of protoxin indicates that it carries three of these 2.5-kd core subunits.

If this glycosylation event and leader peptidase action were the only modifications to M_1-P1 in the in vivo synthesis of protoxin, then endoglycosidase H should convert protoxin to an approximately 32-kd species of the size of the in vitro M_1-$P1_a$ product. Since the deglycosylated protoxin and the product formed in vivo in the presence of tunicamycin are the same size as M_1-P1 preprotoxin, about 2 kd larger than expected (12, 25), either additional modifications occur or the leader peptide removal seen in vitro does not occur in vivo. Other possible modifications might be *O*-glycosylation or palmitoylation, as found for mammalian secreted proteins (120). Evidence is accumulating that the leader peptide remains intact and may be responsible for the binding of protoxin to membranes in vivo (Fig. 5). It has recently been reported (67), on the basis of parallel data, that secretion into the ER of the yeast α mating factor precursor, encoded by the *MFα1* gene, also occurs without removal of the N-terminal leader. It is possible that maturation events unique to precursors of secreted proteins, such as protoxin and pro-α factor, may require the N-terminal attachment of these precursors to the membranes of the secretion pathway. The *KEX2* function (see below) is a candidate for such an event.

Conversion of Protoxin to Toxin in Secretion Vesicles Involves a TPCK-Sensitive Protease

Protoxin is stable in a *sec18* mutant at 37°C, but has a half-life of only 15 min in a *sec1* strain at 37°C, where it is chased into intracellular species of about toxin size. Stability in the leaky mutant *sec7* is intermediate (Table 10). This suggests that cleavage of protoxin to toxin normally occurs in secretion vesicles (25). This was substantiated by the observation that tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK), an inhibitor of chymotrypsin-like proteases, causes accumulation of protoxin in normal, *sec7*, or *sec1* mutant strains at any temperature (Table 10) (25). The maturation of protoxin in secretion vesicles apparently depends on a TPCK-sensitive protease (Fig. 5). Tolylsulfonyl lysyl chloromethyl ketone, the lysyl analog of TPCK and an inhibitor of trypsin-like enzymes, failed to stabilize protoxin in vivo (25).

Effects of *kex1*, *kex2*, and *rex* Mutations

Nonconditional mutations in two nuclear loci, *KEX1* and *KEX2*, were found to prevent expression of killing while allowing normal expression of immunity in a strain carrying a normal [KIL-k_1] plasmid (163). Strains carrying either *kex1* or *kex2* mutations grow slowly, and the *kex2* mutation has a pleiotropic effect on all exocellular proteins and glycoproteins: all are larger and more basic than usual (117). *kex2* strains also fail to mate or sporulate (83). They fail to secrete active α factor (66), secreting instead a massively overglycosylated pro-α factor (67). No such effects are seen in a *kex1* strain, but both mutations may affect some step common to the maturation of unglycosylated secreted proteins. Neither type of mutant strain secretes an active toxin or antigenically cross-reactive material. Protoxin is stable in a *kex1* strain but unstable in a *kex2* strain (Table 10). A *kex1 kex2* double mutant behaves like a *kex1* strain, so that the *kex1*-determined step probably precedes that determined by *kex2*. The protoxin is not stabilized by TPCK in a *kex2* strain (Table 10), suggesting that the *KEX2* step precedes exposure to TPCK-sensitive protease (transfer to vesicles) and that a *kex2* mutation renders protoxin sensitive to a TPCK-insensitive protease system. It is suggested (25; Fig. 5) that *KEX1* and *KEX2* events occur in the Golgi and that protoxin is shunted toward the vacuolar degradative machinery if normal processing is inhibited by a *kex2* mutation. Action of the *KEX2* product may protect protoxin from this shunt and presumably precedes the *sec7* event. Investigation of the biochemical events controlled by these nonessential genes may illuminate aspects of the yeast secretion pathway peculiar to proteins such as protoxin.

[KIL-k_1] strains carrying the *rex1* mutation (150) are suicidal and kill themselves at pH 4.7. This mutation has not been studied extensively. It might result in premature toxin activation, degradation of immunity factor, or modification of the hypothetical membrane receptor for toxin so that it is not protected by the immunity determinant.

SEQUENCE OF THE M_1-P1 PREPROTOXIN GENE

Localization Within the dsRNA of a cDNA Covering Most of the Gene

The in vivo transcripts of M_1-dsRNA were fractionated from the bulk of yeast strain K12-1 polyadenylated RNAs by virtue of their tight binding to poly(U)-Sepharose and were used for oligo(dT)-primed cDNA synthesis (K. A. Bostian, L. Villa-Komaroff, and D. J. Tipper, unpublished data; 10). After guanine-cytosine tailing and insertion into the *PstI* site of pBR322, a 900-bp insert was cloned. This plasmid hybridized specifically with denatured M_1-dsRNA and its transcripts in Northern blots (9). Sequence analysis of the cDNA (10) and comparison with the available data on the terminal sequences of T158c M_1-dsRNA (134, 135) (Fig. 6) demonstrated that one end of the cDNA started with a *PstI* site encoded in the dsRNA at bases 111 to 116, counting the pppG at the 5′ end of the plus strand of M_1-dsRNA as the first base. The cDNA sequence corresponding to the N-terminal 20 amino acids of the α toxin component commences at base 146. Sequence corresponding to the N-terminal 20 amino acids of the β toxin component commences at base 713, in the same reading frame as the α component (10). A single TAG stop codon was found in this frame at bases 962 to 964, and if the β-component comprises the entire C-terminal fragment (Fig. 6), its predicted size (83 amino acids; 9.5 kd) corresponds well to the estimated 9-kd size derived from gel electrophoresis (Fig. 2). In vivo C-terminal processing of the β component cannot be excluded on the basis of present data. Both alternate reading frames have termination codons scattered throughout the sequence.

Only two ATG triplets, at bases 14 to 16 and 99 to 101, exist within the dsRNA and cDNA sequence preceding the α N terminus. Only the former is in frame with the α and β toxin sequences, and labeling of the N terminus of M_1-P1 with leucine and valine (20 and 30 amino acids sequenced, respectively) confirmed that in vitro synthesis initiates at the former (10). The length of M_1-P1 is predicted to be 316 amino acids, with a molecular weight of 34,787, in good agreement with gel estimates.

Domain Structure of the Preprotoxin Processing Sites

A domain structure for M_1-P1 illustrating its potential processing sites is shown in Fig. 7. The cleavages producing the N termini of α and β are trypsin-like (Arg-Glu, Lys and Arg-Tyr, respectively) and are identified as P2 and P4. No Lys or Arg residues exist between amino acid residues 109 and 148, covering the possible size limits of α, except for the Arg-Pro sequence at 134 to 135, a sequence which is cleaved by no known protease type. However, several potential chymotrypsin-type cleavage sites are clustered in the 120 to 132 region where the C terminus of α probably exists. The Trp-Gly bond at 130 to 131 (Fig. 6) is the prime candidate for cleavage by the TPCK-inhibitable enzyme that cleaves protoxin in vivo. This site is located in a generally hydrophilic area of this rather hydrophobic protein and is likely to be exposed on the surface of M_1-P1. To rationalize the stabilizing effect of TPCK on protoxin, and the lack of such an effect for tolylsulfonyl lysyl chloromethyl ketone, we postulate that the C terminus of α is produced by a chymotrypsin-like endoproteolysis (labeled P3, Fig. 7) and that this precedes, and is required for, access of a trypsin-like enzyme to the P2 and P4 sites.

The remaining preprotoxin processing site, P1 (Fig. 7), is that which sequence analysis predicts will be hydrolyzed by leader peptidase during cotranslational transport of M_1-P1 into the lumen of the ER. The in vitro data using dog pancreas membranes, indicating a loss of about 14 amino acids, make it unlikely that the entire 44-amino acid sequence preceding α is removed in this first step. Recent analyses of leader peptidase sites (e.g., reference 109) indicate that all are preceded by a stretch of 8 to 12 primarily hydrophobic amino acids which may favor a β structure in aqueous and an α-helical structure in a membrane environment (32). This stretch is usually preceded by a positively charged amino acid, which may anchor the preceding region on the cytoplasmic side of the membrane (109), allowing the helical hydrophobic region to span the membrane exposing the leader peptidase site to the lumenal surface (32). This site consists most commonly of a β-structure-breaking residue followed, one to three residues later, by an ala-X-ala sequence and a β turn, with cleavage occurring after the second ala residue. Val-X-Ala is the most frequent alternate cleavage site (109). This pattern is followed in the M_1-P1 N terminus by Arg (residue 10), the hydrophobic stretch Val-Leu (residues 12 to 21) terminated by His followed by Val-Val-Ala-Leu (residues 24 to 27). A cotranslational P1 processing site is predicted at the Ala-Leu (residues 26 to 27) bond (Fig. 7). A further 18 amino acids would have to be removed to expose the α N terminus. The discrepancy of the predicted size of the leader peptide (2.9 kd) with that observed in vitro (12) is large, and both estimates of the size of unglycosylated protoxin and experiments on in vivo expression of the cDNA (see "In Vivo Expression of Preprotoxin cDNAs") indicate that, in spite of predictions based on sequence (109), this leader peptide is not cotranslationally removed in yeasts.

All of the AsN-X-Thr/Ser glycosylation sites in M_1-P1 occur in the region between α and β that is excised during processing. This region (γ, predicted to be 103 amino acids; Fig. 6 and 7) is now the candidate for the immunity determinant (Fig. 5). Both α and β contain three Cys residues. No other Cys residues are found in M_1-P1, so a maximum of three disulfide bonds could join α and β. Those shown in Fig. 7 are chosen at random.

Analogies to Insulin and α-Factor Maturation

The maturation of toxin resembles that of insulin (32). Both are processed by removal of N-terminal and internal peptides, leaving two disulfide-linked peptides. Terminal cleavages in both cases occurs within secretion vesicles. A trypsin-like endopeptidase cleaving at Arg, Lys-X is involved in both cases. However, the fragment removed in M_1-P1 is much larger than the insulin C peptides (25 to 35 residues). The insulin C peptide is highly variable in sequence and, it has been suggested (32), mainly serves to ensure that proinsulin exceeds a critical, minimal size for the mammalian secretion pathway. In larger secreted mammalian proteins such as insulin-like growth factor, the C segment is much shorter. A specific function for γ is suggested by its large size, although it may simply be needed to carry sites for glycosylation. Glycosylation may be a prerequisite for channeling of a protein through the secretion pathway in yeasts. Several additional roles for γ may be envisaged: first, after excision, γ may be the immunity determinant; second, as a

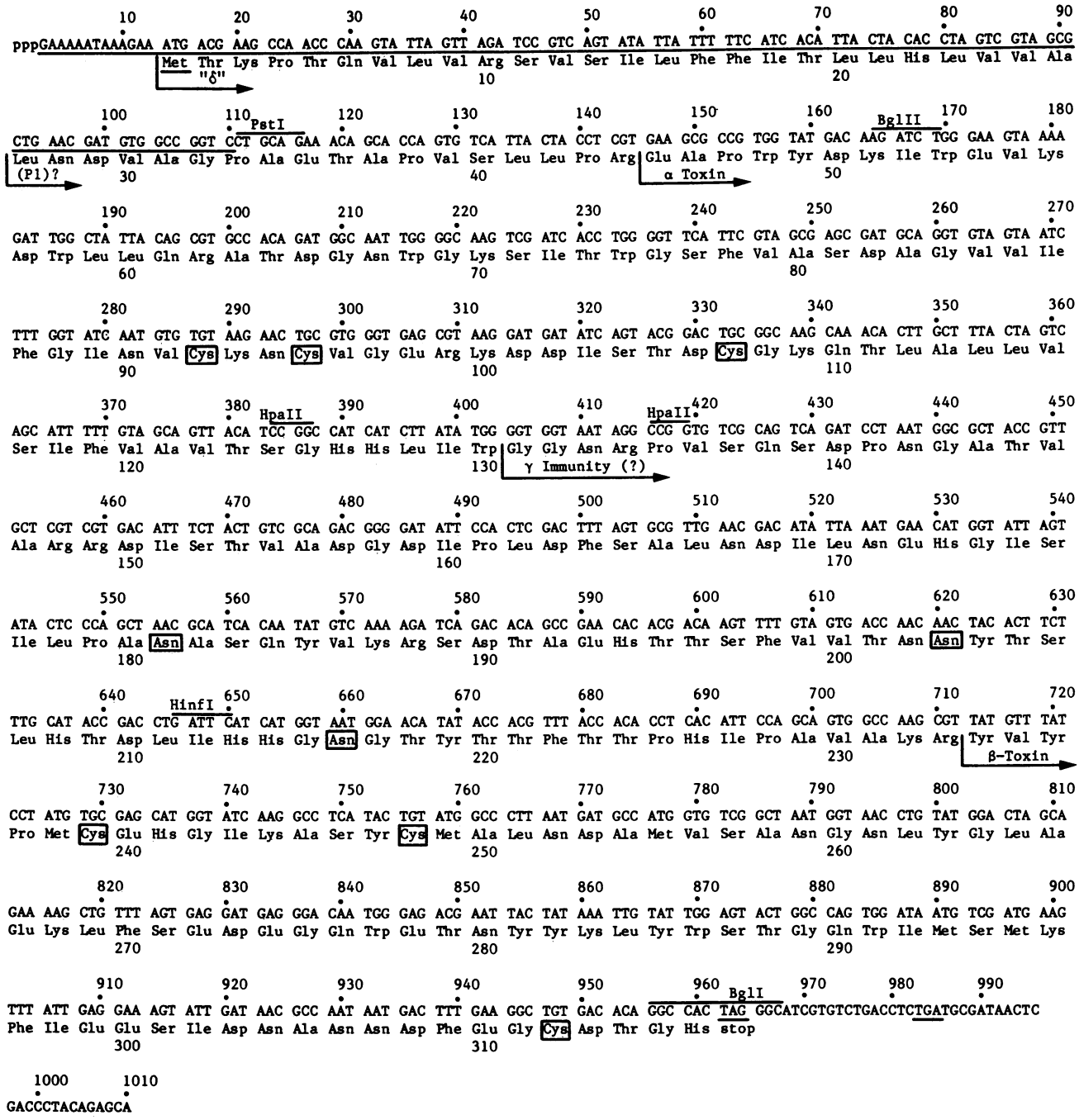

FIG. 6. Sequence of M_1-dsRNA. Sequence from plus-strand bases 1 to 110 is derived from RNA sequencing of the T158c variant (135). The rest of the sequence derives from the cDNA from wild-type M_1-dsRNA of strain K12-1 (10). The start of preprotoxin at "δ" and the N termini of the α- and β-toxin components are shown. The start of the γ segment is hypothetical. The cysteine residues potentially involved in binding α and β subunits and the Asn-X-thr/ser glycosylation sites are boxed.

part of protoxin, it may mask the toxin-active site, protecting the producer cell internal membranes. Third, γ may serve to direct the folding and disulfide bond formation of α- and β-toxin subunits. The membrane association of protoxin is unusual and perhaps unique among precursors of secreted yeast proteins, other than pro-α factor.

Secretion of toxin is strikingly similar to secretion of α factor in yeasts. This factor is a 13-amino acid peptide processed from a precursor which, according to its gene sequence, is a 165-amino acid protein with an N-terminal secretion leader, a 60-amino acid central region containing three glycosylation sites, and four tandemly repeated C-terminal α-factor sequences separated by 6- to 8-amino acid spacer regions (80). Release of the proximal α-factor precursor occurs by cleavage at Lys,Arg-Glu bonds and is followed by action of a membrane-associated aminodipeptidase (66). It has been recently shown (67) that the three sites do become glycosylated in the ER and fail to become elongated.

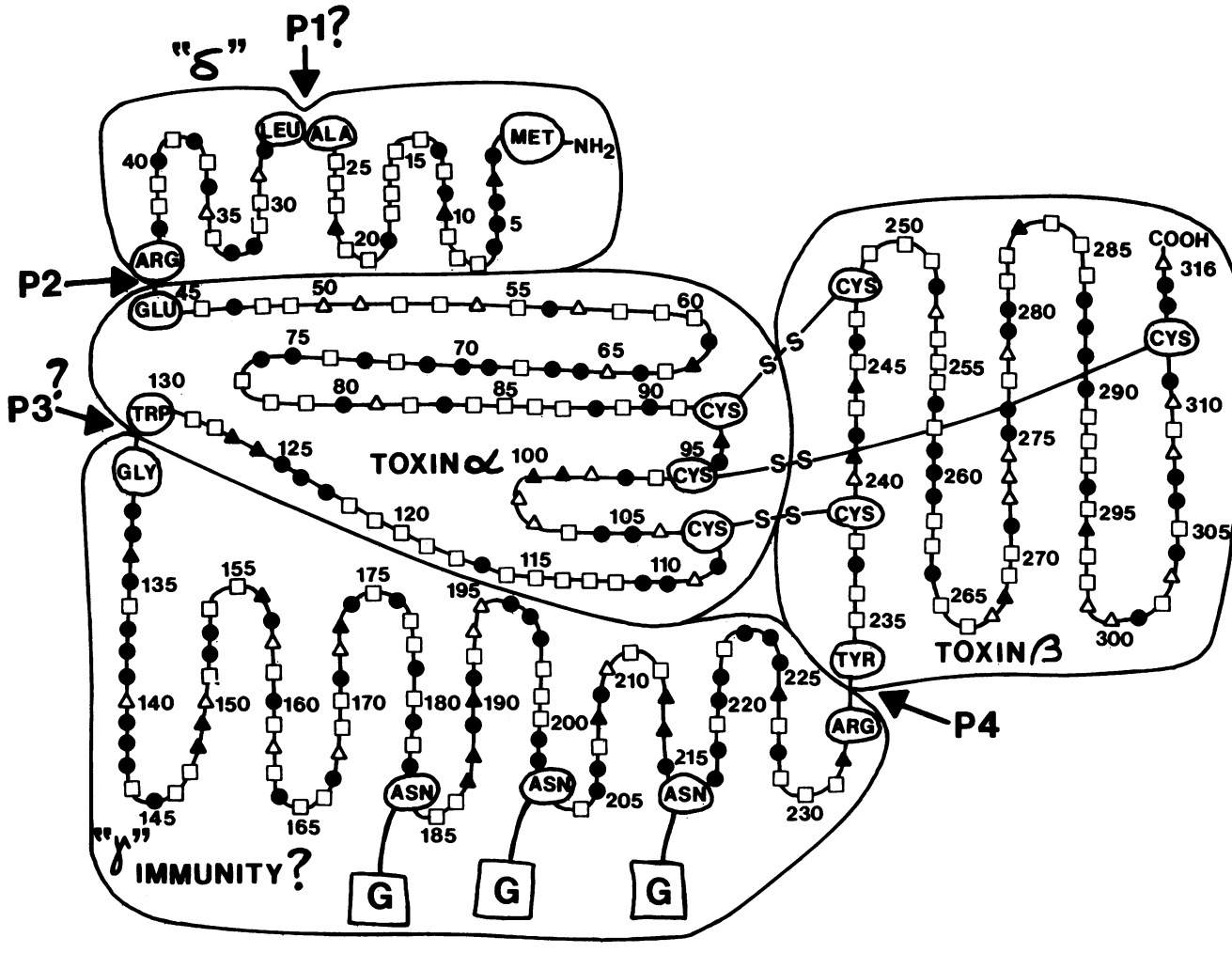

FIG. 7. Schematic domain structure of preprotoxin. Within the N-terminal domain "δ," the hypothetical leader peptidase site (P1) (109) is shown between residues 26 and 27. Processing sites P2 and P4 release the N termini of the α- and β-toxin components. Disulfide bonds drawn between these two subunits are arbitrarily chosen. The subunits are only separable (in sodium dodecyl sulfate) after reduction (25). The chymotrypsin-like site between α and γ is also hypothetical. Glycosylation (G) sites in γ are indicated. Symbols: □, hydrophobic amino acids; △, acidic amino acids; ▲, basic amino acids; ●, other.

Cleavage may initiate in the Golgi and occurs mostly in secretion vesicles. The leader peptide is not removed in the ER. The similarities in organization and maturation of preprotoxin and pre-α factor are obvious, though clear differences in processing exist. There is no evidence for a role for aminodipeptidase in protoxin maturation, and *kex1* does not affect α-mating-type expression. Since overglycosylation of pre-α factor occurs in a *kex2* mutant (67) and normal glycosyl chain extension in invertase apparently occurs in the Golgi (42), this substantiates our prediction of the site of *kex2* action as part of traffic control in the Golgi (25) (Fig. 5). Data on both the *MFα1* and protoxin systems suggest that a nonglycosylated secreted protein in yeasts must have a glycosylated precursor and that maturation of these particular precursors may require retention of the N-terminal leader sequence as a membrane "anchor."

Functional Analysis of the Toxin Subunit Structure and γ

Both α- and β-toxin subunits have a relatively high content of both charged and hydrophobic amino acids. All of the excess charge on toxin responsible for its pI of 4.5 resides in the β subunit. This subunit might, therefore, be supposed to provide the proton binding sites for the cation channels produced by toxin in target cell membranes. However, analysis of the preprotoxin sequence for hydrophobicity, averaged over seven amino acids, shows no long stretches of adjacent amino acids in β with high average hydrophobicity which could correspond to the membrane spanning regions of an integral membrane protein (81). In contrast, the α subunit has two such regions of very high hydrophobicity in its C-terminal two-thirds (preprotoxin residues 72 to 91 and 112 to 127) separated by a highly hydrophilic region which contains four acidic amino acid residues and all three of the cysteine residues of α. It seems likely that spontaneous insertion of this region of α into a membrane might be accompanied by a large conformational shift, with topological eversion of this region, so that previously buried hydrophobic regions interact at their surface with the lipid alkane chains and the hydrophilic region forms a central hydrated core, producing the cation channel. Disulfide binding of β to this hydrophilic region in intact toxin could prevent such a shift, protecting the producer cell membranes against attack.

The killer toxin may resemble the abrin and ricin class of toxins in which receptor binding and toxic domains reside on separate, disulfide-bonded polypeptide chains (106). If so, then β should have affinity for the 1,6β-D-glucan wall receptor, interaction with which may labilize its linkage to α, potentially releasing it for interaction with the membrane (Fig. 5).

Since the toxin is predicted to attack the cytoplasmic membrane via a secondary receptor, then immunity factor might interact with this receptor rather than with toxin. After processing of protoxin, fusion of secretion vesicles with the cytoplasmic membrane would expose the hydrophilic, glycosylated portion of M_1-P1 (now predicted to be the γ peptide) on the exterior of this membrane where the receptor for toxin presumably exists (Fig. 5). Analysis of hydrophobicity (81) has shown that, like β-toxin component, the γ peptide has no long hydrophobic region that would correspond to a membrane-spanning segment. Thus, if membrane association occurs, it is presumed to involve binding to a membrane protein. No means exists for identifying the immunity factor at present, but coupling of appropriate parts of the M_1-cDNA to an expression vector (experiments in progress) should yield a fusion protein which could be used to develop antisera to search for the γ-peptide sequence in vivo. Experiments on in vivo expression (see below) seem to rule out the possibility that immunity factor is derived from a separate reading frame in M_1-dsRNA or from the δ portion of M_1-P1.

Hydrophobicity analysis (81) emphasizes the capacity of the region of δ from residues 10 to 29 to form a membrane-spanning region, consistent with secretion leader functions and also consistent with a role for δ in anchoring protoxin to the membranes of the secretory pathway (Fig. 5). Its ultimate fate is unknown. Fragmentation of M_1-dsRNA at the bubble leads (after denaturation) to in vitro production of a minor 19-kd translation product, derived from the shorter 600-bp terminal fragment (145). This is not produced from intact, denatured M_1-dsRNA. Although it appears, therefore, to be an in vitro artifact, it does demonstrate the presence of a long open reading frame in this region of M_1-dsRNA that might have significance.

IN VIVO EXPRESSION OF PREPROTOXIN cDNAs

The M_1-cDNA sequence has been fused, in frame, to the 5′ promoter, upstream control region, and N-terminal secretion leader peptide of the yeast-repressible acid phosphatase *PHO5* gene (137). After leader peptidase action, a protoxin should be produced containing the last 12 amino acids of the δ region. A sensitive strain transformed with this plasmid remains sensitive in media with high phosphate content, but becomes an immune killer in low phosphate (Fig. 1; S. Hanes, V. E. Burn, D. J. Tipper, and K. A. Bostian, submitted for publication). Only a fraction of the hybrid preprotoxin produced in derepressed cells is converted to toxin. This fraction is processed to protoxin by leader removal and glycosylation. The residual hybrid preprotoxin remains unmodified and may be degraded in the cytoplasm. Inefficiency of processing in this construction may reflect problems associated with leader removal and loss of the N-terminal membrane anchor, inefficiency of insertion of the hybrid molecule into the ER membrane, or overloading of the secretion pathway.

It has recently been reported (123, 125) that a similar cDNA has been prepared from denatured strain T158c M_1-dsRNA, using an oligo(dT) primer presumably initiating within the bubble (16). The cDNA sequence contains an open reading frame virtually identical to that of the K12-1 preprotoxin (Fig. 5), differing in only four base pairs. Two amino acid changes occur (Ile 103 to Ser and Thr 123 to Ala), both in the α subunit, consistent with the observed difference in α-subunit gel mobility (Fig. 2). The other two changes, within the γ sequence, are silent. The clone extends further than the K12-1 cDNA in both 3′ and 5′ directions. At the 3′ end, a 13-bp insert occurs 16 bp downstream of the TAG stop codon (Fig. 5), and an oligo(A)-rich region located 15 to 40 bp downstream of our sequence (Fig. 5) probably indicates the start of the AU-rich bubble. At the 5′ end, it contains most of the preprotoxin leader sequence. It has been fused, at two locations, to the N terminus and 5′ promoter of the yeast *ADH1* gene. Expression of the fused genes should produce preprotoxins with modified N termini: one differs in only two of the first three amino acids, and the other lacks the first nine. Neither modification affects the hydrophobic membrane-spanning region of δ. Plasmids carrying both fusions, transformed into a sensitive strain, produce immune killers (D. Thomas, personal communication).

The results of both promoter gene fusions demonstrate that the portion of M_1-dsRNA cloned contains all of the information needed for toxin production and immunity and strengthen the hypothesis that both functions are contained within M_1-P1 preprotoxin. These results seem to rule out δ as an immunity determinant.

The β killer toxin component and α factor are non-glycosylated C-terminal fragments of glycosylated precursors that carry all of the information used for routing through the yeast protein secretion pathway. Commercial interest in the genes for these precursors derives from the hope that appropriate gene fusions, at the processing sites that result in release of the C-terminal components, may result in secretion of polypeptides of interest, lacking glycosylation or an N-terminal prefix. For toxin, this target for gene fusion is the Lys,Arg-Tyr γ-β boundary.

If a DNA plasmid could be designed to give a transcript with normal M_1-dsRNA termini, then expression of such a plasmid in a sensitive cell containing ScV-L_{1A} might result in synthesis of the cRNA strand and encapsidation. This will be difficult to achieve and not all of the requirements are understood. However, in this way, stable dsRNA plasmids containing and expressing an internal sequence of choice might be synthesizable.

EFFECT OF PLASMID MUTANTS ON TOXIN MATURATION

Two naturally isolated (N1 and N2) and several ethyl methanesulfonate-induced (NLP-1 to -10) neutral variants of M_1-dsRNA have been analyzed for expression of the preprotoxin gene (Table 11) (24). All have a normal-sized M_1-dsRNA, except NLP-3, which had a doublet of M-dsRNAs on agarose gels. Strains NLP-3, -1, and -10 secrete toxin-sized material reacting with antitoxin and retaining 0.1 to 3% of normal toxin activity (Table 11), have normal-sized protoxin of normal half-life, and are presumably missense mutations in the α or β toxin components (24, 25). The significance of the additional M-dsRNA band in NLP-3 is unknown. Strains N1 and N2 fail to secrete toxin activity or CRM, although they produce a protoxin of normal size. This protoxin was unusually stable in strain N1 and presumably has a structural modification rendering it resistant to the TPCK-sensitive vesicular protease. Protoxin stability is no greater than normal in strain N2. It is either destroyed intracellularly, possibly being shunted from Golgi to vacu-

TABLE 11. Expression of preprotoxin genes in M_1-dsRNA mutants (24)

M_1-dsRNA variant	Killer phenotype	dsRNA size (kb)	In vitro translation product (kd)	In vivo protoxin (kd)	Half-life at 30°C (min)	Secreted	
						Toxin activity (%)	Protein reacting with antitoxin
Normal	$K_1^+ R_1^+$	1.9	34	43	25	100	Yes
NLP-1	$K^- R_1^+$	1.9	30	39	25	0	None
-3	$K^- R_1^+$	1.9, 1.8	34	43	25	0.15	Yes
-7	$K^- R_1^+$	1.9	34	43	25	0.12	Yes
-10	$K^- R_1^+$	1.9	34		25	3.5	Yes
N1	$K^- R_1^+$	1.9	34	43	~100	0	None
N2	$K^- R_1^+$	1.9	34	43	25	0	None
SLP-2	$K_1^+ R_1^w$	1.7	34	43	25	100	Yes
SLP-4,6	$K_1^+ R_1^w$	1.9	34	43	25	100	Yes

oles (42), or is secreted and hypersusceptible to exocellular protease.

The most interesting mutant in this class is NLP-1, which can be reverted by ethyl methanesulfonate mutagenesis and is almost certainly, therefore, a point mutation in the M_1-P1 gene (24). It has a normal-sized M_1-dsRNA, but both the in vitro M_1-P1 translation product and the in vivo protoxin are about 4 kd smaller than normal, suggesting the existence of a nonsense mutation about halfway through the β-toxin component. This mutant retains immunity, consistent with the γ peptide being the immunity determinant. No toxin activity or CRM is secreted by this mutant (24).

Several suicidal [KIL-i] mutants were also investigated (24). The most sensitive is killed only by toxin concentrations 100 times those required to kill the same strain after curing of its mutant M_1-dsRNA, so it retains weak immunity. They all produce normal protoxin and toxin (Table 11) and presumably are missense mutations in the γ component of protoxin. One strain (SLP-2) has an M_1-dsRNA of reduced size. This is presumably a consequence of deletion outside of the M_1-P1 gene, unrelated to the $K_1^+ R_1^w$ phenotype.

The presumed sites of these plasmid mutations are indicated in Fig. 3. If the immunity determinant is derived from M_1-P1 and is bound to the outer surface of the cytoplasmic membrane, then the mutant protoxin in strain N1 or the normal protoxin stabilized by TPCK might also accumulate at this site, or even be secreted. However, pulse-labeled protoxin in TPCK-inhibited cells or in strain N1 is not secreted and is not solubilized by protoplasting (25). It was also found (25) that several common proteases mimic in vitro the normal maturation of protoxin by cleaving M_1-P1b to relatively stable fragments of the size of the toxin. However, the accumulated protoxin in neutral strains also fails to become protease sensitive in protoplasts (25). It appears, therefore, to remain intracellular. Since protoxin solubilization requires detergent treatment of ruptured spheroplasts (12), it remains membrane associated as in the wild type under normal conditions.

CODON USAGE IN THE PREPROTOXIN GENE

Extreme bias toward codons recognized by the most abundant yeast tRNAs is shown in codon usage by mRNAs for the major yeast proteins, alcohol dehydrogenase isozyme I, and glyceraldehyde phosphate dehydrogenase (3). Calculation of a codon bias index and comparison with other mRNAs shows a direct relationship between this index and the prevalence of the mRNA (3). The bias ranges from 0.99 for alcohol dehydrogenase I to 0.15 for iso-2 cytochrome *c*. A bias of 1.00 would involve exclusive use of the most abundant tRNAs (underlined in Table 12), whereas a bias of 0 would represent a random choice between the tRNAs available. The bias calculated for the M_1-P1 gene, whose codon usage is shown in Table 12, is −0.04, indicating a slight bias against the most common tRNAs. This is clearly consistent with the low abundance of the preprotoxin mRNA, as we have determined (Table 6) (10).

LINEAR DNA KILLER PLASMIDS OF *K. LACTIS*

K. lactis strain ATCC 5356 contains two linear dsDNA species, pGKl-1 (8.9 kb) and pGKl-2 (13.4 kb), each in about 50 to 100 copies (53). pGKl-1 determines the production of an exocellular toxin composed of 27- and 80-kd glycoprotein subunits (51). The plasmids can transform *K. lactis* conferring killer phenotype (40). The toxin kills a wide range of yeasts, including *Kluyveromyces*, *Saccharomyces*, *Candida*, and *Torulopsis* sp. strains, and has been shown to inhibit adenylate cyclase in *S. cerevisiae* (51), possibly mimicking the effects of yeast α pheromone. Killers are specifically immune and both immunity and killing are cytoplasmically inherited in *K. lactis*. Transfer of both plasmids to *S. cerevisiae* by protoplast fusion or transformation confers the *K. lactis* killer and immunity phenotype on the recipient cells (52). The pGKl-1 plasmid is stable in ρ0 *S. cerevisiae* cells,

TABLE 12. Codon usage in the killer preprotoxin gene[a]

Phe	UUU	9	Ser	UCU	2	Tyr	UAU	8	Cys	UGU	3
	UUC	2		UCC	2		UAC	3		UGC	3
Leu	UUA	7		UCA	6						
	UUG	3		UCG	4				Trp	UGG	9
Leu	CUU	3	Pro	CCU	5	His	CAU	7	Arg	CGU**	6
	CUC**	2		CCC	0		CAC	4		CGC	0
	CUA	6		CCA	5	Gln	CAA	4		CGA	0
	CUG**	4		CCG**	2		CAG	3		CGG	0
Ile	AUU	8	Thr	ACU	3	Asn	AAU*	11	Ser	AGU*	8
	AUC	7		ACC	7		AAC	8		AGC	2
	AUA*	5		ACA	10	Lys	AAA	3	Arg	AGA	2
Met	AUG	6		ACG	5		AAG	10		AGG	1
Val	GUU	4	Ala	GCU	5	Asp	GAU	11	Gly	GGU	11
	GUC	5		GCC	8		GAC	10		GGC	8
	GUA*	8		GCA	8	Glu	GAA	8		GGA*	3
	GUG	8		GCG	4		GAG	6		GGG	1

[a] Data are from the preprotoxin sequence shown in Fig. 6. The codons recognized by major tRNAs and almost exclusively used by major mRNAs are underlined. The codons used by preprotoxin and only in the least abundant of eight mRNAs studied (3) are indicated by *. Those used by preprotoxin but not even in this mRNA are indicated by **.

but becomes unstable if mitochondria are reintroduced to the *S. cerevisiae* killers (52). This phenotype bears some resemblance to the effect of a *mak10* mutation on ScV-M_1 and suggests incompatibility between mitochondrial DNA and the *K. lactis* plasmids in *S. cerevisiae*. No such effect is seen in the petite-negative *K. lactis* species.

Spontaneous or induced nonkiller mutants of both pGKl-1-containing *K. lactis* and *S. cerevisiae* killers fall into several classes (101, 147, 148): loss of both plasmids, loss of only pGKl-1, central or terminal deletions of pGKl-1, and point mutations in this plasmid. Some of those with central and point mutations are neutral and retain the immunity determinant. An open reading frame for 1,146 amino acids in pGKl-1 presumably codes for a preprotoxin (52), as in the *S. cerevisiae* M-dsRNA system, but no data on intracellular toxin precursors and transcripts, or on the mechanism of toxin secretion, have been published. *K. lactis* chromosomal mutations equivalent to the *S. cerevisiae kex* mutations have been isolated (147) and may affect toxin maturation.

The role of pGKl-2 in pGKl-1 maintenance remains conjectural. The two plasmids are not encapsidated and bear no homology either to each other or to nuclear or mitochondrial DNA.

The termini of pGKl-1 have 202-bp inverted repeats. Similar 182-bp repeats are found in pGKl-2 (51). They bear some resemblance, therefore, to the telomeres of yeast chromosomal DNA and are presumably essentially involved in replication.

The stability of these plasmids enhances their potential as cloning vectors. Restriction endonuclease maps are available (148) and sequencing is in progress. Hybrid plasmids between pGKl-1 and the *S. cerevisiae URA3* gene stably transform uracil-requiring *K. lactis* giving URA^+ progeny, but the reisolated plasmids are circular rather than linear, as anticipated (40).

dsRNA KILLER SYSTEM OF *U. MAYDIS*

U. maydis, the smut fungus of maize, causes callus formation in infected tissues and is one of the many plant-pathogenic fungi (37). Killers of three different immunity specificities (P1, P4, and P6) have been found among natural isolates; each type kills the other two but is immune to its own toxin (72–74, 168). The toxins do not kill *S. cerevisiae*. Each killer type is associated with cytoplasmic dsRNAs individually encapsidated in VLPs (71, 75), including at least one representative of each of three size classes: heavy (H), 3.6 to 6.2 kb; medium (M), 0.92 to 1.7 kb; and light (L), 0.35 to 0.36 kb. The distribution of dsRNA species of similar size in representative killer strains are presented in Table 13. M2(4) and M2(6) refer to the species of M2 size found in P4 and P6 killers, respectively. Since the P6 killer strain carries only H1, M2(6), and L, and variants of P1 and P4 killers are known to lack several of the H components listed (71), it appears that the minimal requirements for a killer is an H and an L species plus the appropriate M species: M1(1) for P1, M2(4) or M3 for P4, and M2(6) for P6 killers.

The *U. maydis* virus (UmV) VLPs have a single major 75-kd capsid protein (76) which is probably encoded by one or more of the H dsRNAs (S. Simon, M. Gorecki, and Y. Koltin, Genetics **97**:s76, 1981), since it is found both in strains containing only H1 and in related strains containing only H3 and H4. Toxin production (76) and immunity (108) segregate with the M-dsRNAs (Table 13) and in vitro translation of these dsRNAs produces a 19-kd product antigenically related to the 12-kd toxin (Simon et al., Genetics **97**:s76, 1981). The analogy to the ScV system is obvious; however, the variety of H and M species and the presence of L species are unique.

TABLE 13. UmV dsRNA sizes and distribution[a]

dsRNA	Size (kb)	P1	P4	P6
H1	6.2	+	+	+
H2	5.0	+	+	
H3	3.8		+	
H4	3.6		+	
M1 (1)	1.7	+ (K)		
M2 (6)	1.2			+ (K)
M2 (1, 4)	1.1	+	+ (K)	
M3	1.1	+	+	
L	0.36	+	+	+

[a] Sizes are approximate (45). The species determining killer phenotype are indicated by (K). Numbers in parentheses indicate the killer type in which dsRNAs of similar size are found.

Northern gel and heteroduplex analyses of the strains listed in Table 13 demonstrate that the L species in P1 (P1-L) is entirely derived from one terminus of M1(1), whereas P4-L is similarly homologous to one end of M2(4) or M3 (M_2 and M_3 were not resolved) and P6-L is homologous to one end of M2(6) (45). The significance of the existence of these small (0.36-kb) redundant dsRNAs, derived from the killer determinant dsRNAs, is unknown. L is clearly too small to encode the 19-kd toxin precursor, although it could encode a 7-kd fragment of it, possibly related to immunity.

The Northern gel analysis also demonstrated partial homology between the killer determinants M1(1) and M2(4) + M3. Neither is homologous to M2(1) and neither has homology to M2(6) (45). This is consistent with the partial overlap of immunity specificities between P1 and P4, but the lack of overlap of either with P6 (72).

Probes of H1 from P1, P4, or P6 reacted with the H1 species of all three types, showing relatedness but not identity. The same relationship exists between the H2 species of P1 and P4 but, surprisingly, H1, H2, and H3–H4 species are not related, even within the same strain (45). It seems likely, therefore, that the capsid peptides presumably encoded by these various H species will also differ, like those encoded by the ScV L_{1A}-, L_{2A}-, and L_{BC}-dsRNAs. A complexity at least as great as that seen in the ScV-L system is apparent in *U. maydis*.

Limited 3′-sequence analysis has been performed (45) and demonstrates that, as for the L_{BC}-dsRNAs of *S. cerevisiae*, the UmV dsRNAs have either 3′-terminal A-OH or G-OH residues which are presumed to be unpaired and added post-transcriptionally.

EFFECTS OF dsRNA MYCOVIRUSES ON PATHOGENIC FUNGI

As stated in the Introduction, killer phenomena in yeast presumably have a substantial impact on competition between related species and (less frequently) between yeast genera for their favored ecological niche. This may also apply to pathogenic species which compete for parasitization of host tissues. The effects of killer plasmids on virulence of animal pathogenic species such as *Candida* and *Cryptococcus* appear to be minimal, and their presence and toxin susceptibility are only important for epidemiological studies at present (91, 92, 113). However, effects of mycovirus infection on the virulence of plant-pathogenic fungi of major economic importance have been reported and are under investigation.

Whereas the killer system in *U. maydis* is not known to affect virulence, cytoplasmically inherited dsRNAs found in certain strains of *Endothia parasitica* profoundly affect the virulence of their host (140). *Endothia parasitica* was introduced into North America early in this century, probably from the Orient, and virtually eliminated the American chestnut over the next 50 years. A similar pattern occurred in parallel in Europe; however, hypovirulent strains causing a chronic, nonlethal infection developed there. These strains carry three to five dsRNA species in the size range of 5 to 11 kb, and cytoplasmic transmission of these species correlates with hypovirulence. They have not been further characterized. Although isolated in particles, these particles appear to be membrane vesicles rich in carbohydrate and lipid, but low in protein content, rather than encapsidated VLPs (140).

Rhizoctonia solani, a broad-range pathogen for many economically important crops, has also been found to contain dsRNAs associated with hypovirulence (140). *H. victoriae*, the causal agent of Victoria blight in oats, is itself susceptible to a "disease" characterized by stunted mycelial growth, poor sporulation, generalized lysis, and distorted mycelial cell walls (48). Transmission of these characteristics, which markedly reduce virulence for plants, is associated with dsRNA-containing VLPs. Serologically unrelated 190S and 145S VLP species are found, containing one and four dsRNA species, respectively. Healthy, virulent *H. victoriae* isolates contain no dsRNA or only the 190S VLPs. The 145S species is the presumed agent of the disease of the fungal mycelium and appears to be unstable (48). A low (1%) but significant rate of disease transmission was observed on mixing normal *H. victoriae* protoplasts with VLP preparations containing both the 190S and 145S species (48). The 145S species may be a satellite virus of the 190S species, analogous to the relationship between the ScV-M and ScV-L_A species of *S. cerevisiae* VLPs.

Within the ScV system, killer dsRNA-containing VLPs have been introduced into commercial fermentation strains (160) by mating or cytoduction, to guard against the type of takeover by wild-type killers first observed in sake and beer fermentations (65, 88).

EVOLUTION OF THE KILLER SYSTEM

As pointed out by Holland et al. (61), RNA genomes are found only in viruses parasitic on cells with DNA chromosomes. They may represent relics of a pre-DNA biosphere or self-replicating transcripts of a DNA progenitor. They comprise most of the disease-causing viruses of eucaryotes and profoundly affect the survival and evolution of their hosts.

The error-correcting mechanisms inherent in the DNA synthetic apparatus (e.g., proof-reading exonuclease and marking of the template strand by methylation) are not believed to exist in the various RNA-dependent transcriptases, all of which have virus-encoded components, and evolution of RNA genomes by replication error is rapid, so that selection for replication efficiency is of major importance to the survival of these parasitic genomes. Thus, the frequency of spontaneous temperature-sensitive vesicular stomatitis virus mutants is about 1 to 2% and silent mutations must occur at similar frequency, 10^{-4} to 10^{-5} per nucleotide per replication (61).

The production of defective-interfering particles, dependent on parental genomes as helper viruses (63), is a graphic illustration of both the frequency and constraints on RNA virus mutation. In persistent infection by a mixture of vesicular stomatitis virus and defective-interfering particles, mutation and evolution may continue toward a state in which the virus is almost incapable of forming infectious particles. If this ability were lost, a plasmid-like state reminiscent of the mycoviruses would result. Mycoviruses might have arisen by a similar mechanism, although the absence of known infectious viruses in fungi may be related to the excellent protection provided by their cell walls.

Whatever their origin, mycoviruses such as ScV-L_A now exist in apparent amicable equilibrium with their host cells, producing at least one component essential for their own survival, but dependent on many host gene products. Mutation to runaway replication has never been observed, probably because of potential lethality and the absence of transmission. Perhaps more surprising is the stability of ScV-L "infection" implied by the absence of ScV-free segregants, in the absence of known physiological advantage conferred by ScV-L on R^- K^- host cells. These dsRNA genomes seem to be peculiarly stable. The origin of the dsRNA species conferring male sterility on *Vicia faba* (49) is also unknown.

The origin of the ScV-M killer plasmids, satellites totally dependent on ScV-L_As, is a different problem. They presumably appeared by acquisition of genes unrelated to dsRNA maintenance after the L-dsRNA systems were established. Although they negatively affect ScV-L copy number and must share some replication components, the lack of homology between L- and M-dsRNAs suggests that, if they have a common origin, their divergence is ancient. Perhaps rare nuclear RNA recombinational events (splicing errors?) lead to insertion of information from DNA genes into ScV-L transcripts. The complete killer phenotype would probably have had to evolve as a DNA system before such an event, since toxin production and immunity would presumably be inseparable. Both may have derived from a single transmembrane protein complex involved in controlling cytoplasmic ion concentrations. Perhaps further investigation of the modes of action of toxin and immunity may lead to recognition of normal cellular counterparts of their functional components.

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